

The post-Marx dearth of capital

The collapse of communism, the antithesis of capitalism, in Eastern Europe, and its economic failure in the Soviet Union, has brought to light a paradoxical circumstance — the world is dangerously short of capital.

KARL MARX, in retrospect, had a very simple view of life. People would work for the benefit of their communities, benefitting personally, like all others, to the extent the community saw fit. In stalinist times, marxism was an excellent recipe for what would elsewhere have been called capital formation. It was simply necessary to adjust the ratio of those employed to build factories and on other long-term projects to those employed to provide the community as a whole with the necessities of life. The growth of heavy industry in the Soviet Union during the 1930s was a remarkable proof of the efficacy of the process. One of the reasons why communism has come unstuck is that too little was left over to satisfy the legitimate aspirations of the people for a better life. (The accompanying lack of liberty is something else again.) It is a savage irony that most of this past capital investment is inappropriate to modern needs: it has been wasted.

So Eastern Europe and the Soviet Union are on the look-out for more capital, but at the least auspicious time. Most other places are also crying out for the same stuff. Where to find it, and how to recognize it when you do? Capital is nothing but money, and thus indistinguishable from what people use to buy train tickets or dishwashers. The difference is that capital is the money left over after people have spent what they wish to spend on their immediate needs and have paid the interest on their debts. At the personal level, capital is a person's savings. At the national level, the maximum indigenous rate of capital formation is the aggregate of these savings less the net cost of keeping the government in business. People, businesses or governments needing more capital than they have saved must borrow it from the banks and the other agencies of what is now an international market in capital.

Lip reading

The obvious difficulty, for Eastern Europe and the Soviet Union, is that the natural sources of capital are in dire need of the stuff themselves. In the United States, President George Bush has had to go back on his memorable election promise that there would be no new taxes (devices for enforcing savings) in the hope of an agreement with the Congress that the annual budget deficit will be reduced by two-thirds, or by a cool \$100,000 million. In Britain, where the personal savings ratio has also fallen dramatically in the past decade, the hapless

Minister of Transport, fresh from ruling that the country cannot afford a high-speed rail link to the Channel Tunnel or even a new subway line across London, let slip at the weekend that it cannot afford a new aircraft runway in South-East England either.

But not everybody is strapped for capital. In several countries, Japan and West Germany particularly, both the personal and the corporate sectors are saving hard, while corporate sectors elsewhere have money left over after paying dividends to their shareholders. Where does that finish up? During the past decade, it has been fashionable to invest in the United States, sometimes by buying companies outright (to the frequent chagrin of the locals), sometimes by lending directly to the US government. The reunification of Germany will seriously disturb that habit. The Bonn government, for example, estimates that reunification will cost it DM80,000 million in 1991, most of which will be borrowed from the banks. German companies will invest several times as much in the East. What will be left over for other people?

That is where the crunch comes. When capital is scarce, its price increases, which is why interest rates are likely to remain high for a long time. Outside the umbrella of united Germany, Poland, Czechoslovakia and Hungary will find themselves paying through the nose for private investment — and may consider themselves to have been exploited as a consequence. The imaginative idea of a European investment bank, due soon to be a reality, should blunt the edge of that discontent, but not by much. There will be two losers — the United States, where the economy seems nicely poised between boom and recession, and the poor countries of the world, which will be now more than ever dependent on government-backed assistance, either bilateral or through institutions such as the World Bank. Either way, it is a dangerous prospect for the rest of us. □

Genome goes cool

The human genome project deserves a better hearing from the US Congress than it is likely to be given.

THERE is no good reason why the US House of Representatives should have soured in its view of the Human Genome Project. It would be different if the House were



zealously cutting every possible budget item so as to bring the federal deficit under control, but that is plainly not the case. The Senate may be able to insist that the project should not be made to rub along, in the year beginning on 1 October, on roughly half of what it needs from the National Institutes of Health, but it is not well placed to make that point (see page 309). The excuse that the earmarking of research funds for a particular project undermines the primacy of the principal investigator sounds like a deliberate misunderstanding; that the project would consist of a great deal of production-line research work has been made clear from the start.

The essence of the case for the genome project is that the sequence of the human genome will not be compiled by accident. Despite the speed with which particular human genes are being identified and their nucleotide sequences established, the indefinite continuation of that process will never yield the nucleotide sequences of the apparently boring bits of the genome that lie between identified genes, whose evolutionary significance may nevertheless be great. And there is no prospect that even the present rate at which human genes are being identified will make the human genome the analytical tool it might be — a databank of which it is possible to ask interesting questions. Unless the compilation of that databank is commissioned, it will never exist.

That is the case against the position taken by the House Appropriations Committee, but it should be acknowledged that the genome project's own posture is not without its inherent problems. It is caught in a dilemma created by its own existence. The prospect of a deliberate effort to sequence the human genome has stimulated a plethora of new techniques, further complicating the development of a coherent strategy. Sensibly enough, the project plans to cut its teeth on the sequencing of a smaller genome, while constructing a physical map of the human genome. The plan to use NIH money to commission some centres now will give the project the luxury of choice at a later stage, when the responsibility for the long haul will have to be shouldered by a subset of them. Congress's long experience with military projects should be a warning against parsimony at this early stage. But that is a less clear message than those to which congressmen are used.

The military analogy can be taken at least one step further. Making a telling case to the US Congress is a matter of giving the impression that the objective is nicely crystallized without inhibiting the freedom to change course radically at a later stage. Generals pleading for funds for exotic new weapons systems have learned to give the Congress the impression of always knowing exactly what they want; if they later change their minds, they usually manage to get away with it. One consequence of what is called the peace dividend is that there will be experienced Pentagon negotiators out of work. It might suit the genome project's book to hire one or two of these experienced operators to get the appropriations bill through Congress. □

Television violence

Evidence that television breeds violence remains as elusive as ever.

WHETHER or not television has the inherent power to incite violence in its consumers, the square-eyed hordes seem to have more personal and cultural 'fortifications' than is usually supposed. The annual research review of the Broadcasting Standards Council, published last week, leapfrogs the debate in Britain over whether the content of television should be regulated, calling instead for a general and dispassionate recognition of the complexity of the issue and, crucially, of the viewer.

The report, the council's first since it was set up in 1988 on an ominous 'pre-statutory' basis, takes a refreshing approach to what has become a rather turgid issue. Instead of trying to account for the role of television in the violent behaviour of violent criminals, the council's researchers, led by Dr David Docherty of the London School of Economics, has asked how television may affect the attitude of the viewing public towards violence itself.

For their main survey, the researchers asked just over 1,000 British viewers how they felt about violence in Britain today — a survey which, as well as providing a useful gauge of public sentiment, allowed the researchers to test for correlations between people's television consumption and their overall attitude towards violence. For instance, do heavy television consumers feel differently about 'legitimate' violence, such as capital punishment and armed police action, than their more abstemious counterparts, and are they, in general, less secure about their own safety?

For the most part, the research shows no significant differences between the heavy viewers and the light on such questions. In fact, the issue that provokes the clearest discrepancy between the two groups — the arming of police in various confrontational situations — shows heavy television viewers to be cooler-blooded in every instance than their library-going and apple-bobbing compatriots. The report concludes that there is a qualitative sifting, on the part of the viewer, of violent images into different categories of palatability. Docherty suggests that it is the extent of the viewer's involvement in the fiction that is the determining factor in these classifications. Television violence that somehow involves the viewer — whether by eliciting an intellectual response or simply by reflecting life in Britain — is far more likely to upset than that which makes fewer claims to realism. In short, the report holds that the context of fictional violence is at least as important as its substance when it comes to earning the label "unacceptable"; and, whether or not television has the power to incite people to violence, viewers are more actively discerning than some proposed legislation gives them credit for. The research does not provide justification for the restrictive regulatory regime that some would like. □

First UK trial of AIDS vaccine approved

- First step is cautious but safe
- Twenty volunteers to test prototype

London

THE first British trial of a prototype AIDS vaccine was given the go-ahead by the UK's Medical Research Council (MRC) last week.

The phase I trial, which will be carried out under the aegis of the MRC's AIDS-related research programme, is to begin in September at London's Hammersmith Hospital, where a candidate vaccine known as p24-VLP will be administered to 20 healthy seronegative volunteers. Their immune response to the vaccine will be monitored for up to a year before a decision about further trials is made.

Developed by Oxford-based British Biotechnology Limited, p24-VLP consists of a fragment of an HIV protein known as p24 presented on the surface of a newly-emergent vaccine carrier known as a virus-like particle (VLP). Although not infectious, VLPs possess some of the basic elements of retroviruses, and can be prepared easily in a pure state from genetically engineered yeast cells. Researchers at BBL have shown that VLPs are able to carry proteins that induce HIV-specific immune responses in animals.

News of the trials comes almost three years after the start of the first clinical trial of a prototype AIDS vaccine in the United States. Jane Cope, who is orchestrating the MRC's AIDS vaccine strategy, says that the MRC "didn't want to rush into human studies" without first laying the foundations of a well organized trial system.

But aside from caution, there may be other reasons why the announcement of the first UK trial has been slow in coming. One factor, according to Alan Kingsman, director of research at British Biotechnology Limited, is the dearth of small biotechnology companies in Britain: "The big companies haven't been keen to take it [vaccine development] on", he says. Jonathon Weber of the Hammersmith Hospital, who will be in charge of the clinical trials, notes that most US trials have focused simply on the response of the human immune system to large quantities of crude HIV proteins or peptides. The British trial, however, has a different emphasis: to test the feasibility of using VLPs as novel carriers for 'immunogenic' HIV peptides in humans.

Caution is also evident in the choice of p24 as the vaccine's immunogenic component. Most vaccines tested so far have used the HIV envelope proteins gp160 and gp120, on the grounds that these proteins are exposed on the surface

of the virus and so should be strongly immunogenic. In the past, however, questions have been raised about the safety of envelope-derived vaccines, which some researchers have suggested could act to suppress the immune system. No such concern has been expressed over p24, largely because, being a core protein of HIV, it does not participate directly in the virus's interaction with T cells.

Kingsman and Weber agree that p24 alone is unlikely to provide protective immunity against HIV. They view the trial as the first step towards developing a vaccine in which VLPs carry a 'cocktail' of HIV immunogenic protein fragments. The p24 protein is known to stimulate helper T cells in animals and so might play a part in creating protective immunity.

Although trial volunteers will produce antibodies to HIV, the MRC has given assurances that no one will be mistakenly thought to have HIV infection, and so volunteers should have no difficulty obtaining life assurance or mortgages on medical grounds.

The researchers were coy about how they intend to recruit volunteers, but it is unlikely that they will need to call on members of the public. **David Concar**

COLD FUSION

Second round

Washington

FIFTEEN months after the appearance of their first paper announcing the discovery of "cold fusion", Martin Fleischmann and Stanley Pons have published a second paper, 56 pages long, in which they give a full account of the electrochemistry and calorimetry behind their claim that electrolysis of heavy water with palladium cathodes generates more heat than conventional chemistry and physics can explain. The paper, written by Fleischmann, Pons, M.W. Anderson, L.J. Li and M. Hawkins, all of the Department of Chemistry at the University of Utah, appeared in the 25 July 1990 issue of the *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, the same journal in which a short paper by Fleischmann, Pons and Hawkins appeared on 10 April 1989.

According to an announcement by Elsevier Sequoia, the journal's publisher, the new paper addresses only questions of electrochemistry, and contains no information on the emission of nuclear particles or radiations which would be expected if "cold fusion" reactions are indeed occurring. The accuracy of the calorimetric results is demonstrated, Elsevier says, by comparisons of palladium-heavy water cells with control experiments using palladium electrodes in light water and platinum electrodes in heavy water. In the absence of direct evidence for nuclear processes in the cells, the implication that fusion is responsible for the excess heat is drawn "by elimination". **David Lindley**

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Light speed — a solar-powered car leaves engine troubles behind on its way to fourth place in the General Motors Sunrayce earlier this month. The 1,641-mile race for vehicles powered by sunlight was won by an entry from the University of Michigan. (AP)

Heading for the nineties

Washington

US neuroscientists' attempts to turn a Congressional resolution designating the 1990s the 'Decade of the Brain' into a financial reality gained a little help last week when US president George Bush signed a proclamation calling on the decade to be observed with "appropriate programmes", and the First Lady, Barbara Bush, opened a two-day meeting intended to publicize the start of the decade.

Both the National Institute of Mental Health (NIMH) and the National Institute of Neurological Disorders and Stroke (NINDS), the two institutes of the US Department of Health and Human Services most likely to benefit from increased efforts in neuroscience, have already released plans laying out what could be achieved in the decade.

The report from NINDS, released last month, also reveals what the decade might cost — for NINDS alone an initial increase of \$221 million above the administration's \$500 million budget request, followed by a further rise of similar size two years from now.

Funding requests on this scale need a compelling argument if they are to make it through Congress. At last week's 'Decade of the Brain' meeting at the National Academy of Sciences, the logic came in two parts. At the meeting were a selection of the finest neuroscientists working in the United States, speaking in a programme that ran from work on ion channels to neural-network computing. These scientists delivered the first part of the message — that neuroscience is now a mature discipline in the sense that it contains areas in which it is fairly evident what needs to be done and, broadly speaking, how to do it. What that means is that Congress and president can be sure that increased investment will translate directly into increased results.

Areas where the prospects for rapid progress are good were listed by NIMH in a document given out at the meeting: they include the identification and cloning of the genes responsible for schizophrenia, manic-depressive illness, Alzheimer's disease and other mental illnesses; the detailed mapping of where various neurotransmitters and receptors act within the brain; the use of computer-analysed brain activity measurements in improved diagnosis of brain disorders, and use of models of receptor structure to design and synthesize drugs that could help treat conditions, such as schizophrenia, that are thought to be due to defects in neurotransmission.

The second part of the logic, presented most graphically by Lewis Judd, director of the National Institute of Mental Health is that mental illnesses are a gigantic

burden for the health-care system. Judd points out that some 15–20 per cent of people are affected by some form of mental illness each year. The Congressional proclamation estimates that in the United States, the "treatment, rehabilitation and related costs of disorders and disabilities that affect the brain represents a total economic burden of \$305,000 million annually". Investment in basic research to produce more effective forms of treatment thus has the potential for generating a large return.

"If you combine the costs of 50 million people a year suffering from mental disorders with a science that is growing exponentially then you have a potentially convincing argument that we're hoping will carry the day, and that funding will flow from it", says Judd. He and his fellow institute directors are not themselves permitted to lobby for increased funds; the job, he says, will have to be performed by the many patient groups (the Cerebral Palsy Foundation, the Epilepsy Foundation and so on) as well as the various professional and scientific societies. A first move of this kind was announced at the conference by David Mahoney, a director of the Dana-Farber Foundation. The foundation is to set up an institute to lobby for support for the decade, and a \$25,000-a-year prize to be offered each year of the decade to an outstanding neuroscientist.

Are president and Congress going to be persuaded to support the decade? The House of Representatives has already given mention of the Decade of the Brain as an "area of special concern" in its 1991 appropriations bill for the Department of Health and Human Services, but there will be very little new money available this year. A panel discussion at the conference which included Allan Bromley, White House Science Advisor, James Mason, Assistant Secretary for Health, and Frederick Goodwin, administrator of the Alcohol, Drug Abuse and Mental Health Administration, raised few hopes that riches were about to be showered on neuroscientists. Although steps are being taken to set up a coordinating committee within the White House, most talk at the conference was of the gloomy prospects presented by the growing federal budget deficit.

The next public event in the Decade of the Brain will be the start of a Library of Congress lecture series sponsored by the National Institute of Mental Health. The lectures will continue throughout the decade, with clusters of six lectures each autumn and spring. The first lecture, appropriately enough, will focus on the cost to the United States of brain-related disorders.

Alun Anderson

Pyrrhic victory in South Pacific

Sydney & Tokyo

UNDER pressure from Australia and other South Pacific nations Japan announced last week in Canberra that it will suspend drift-net fishing in the South Pacific a year earlier than required by a United Nations resolution. But the decision will have little overall effect on Japan's drift-net operations, which are based largely in the North Pacific.

Drift-net fishing, which employs sets of nets up to 60 km in total length to catch tuna and squid, has been condemned because it kills other marine life indiscriminately, in particular turtles, seabirds and dolphins. Last year the United Nations adopted a resolution, endorsed by Japan, that bans the technique in the South Pacific from 1 July 1991 and worldwide a year later, unless it is deployed using proven management techniques.

Japan's suspension will come into effect in October, the beginning of this year's season. But, as pointed out by Peter Gill of Greenpeace, "Japan has withdrawn less than thirty boats from the [South Pacific] region while they maintain over one thousand boats in the North Pacific". The South Pacific decision is "chicken feed" compared with the North, Gill says, and he doubts that the UN resolution will bring about a worldwide ban by 1992.

Tania Ewing & David Swinbanks

Wildlife response to the nets

Washington

THE US National Marine Fisheries Service (NMFS) finally agreed earlier this month to release some of the data they have collected on the impact of drift-net fishing on marine life after a meeting between Congresswoman Jolene Unsoeld (Democrat — Olympia) and the director of NMFS. Unsoeld has been campaigning for a ban on the technique. The data come from a survey conducted last year by US, Canadian and Japanese observers on board some of the 470 licenced Japanese drift-net boats that catch squid in the North Pacific during the summer months.

Observers looked at a total of 1,402 'sets' of the net — about one fiftieth of the total — in each case examining about three-quarters of the catch. They found that the nets had caught 208 fur seals, 914 dolphins, 22 turtles, 539 albatrosses, 8,536 shearwaters, 25 puffins (an endangered group) and 17 storm petrels. NMFS officials are not yet ready to say what impact this level of mortality might have on the total population of North Pacific sea mammals, turtles and birds. A more detailed survey is being carried out this year with three times as many observers aboard the fishing boats.

Alun Anderson

Spanish to step in?

London

THE UK Science and Engineering Research Council (SERC) last week failed to decide whether to collaborate with Spain or with the United States in a project to build what will be the most powerful optical-infrared telescope in the Northern Hemisphere.

But SERC's indecision may now tip the balance in favour of the Spanish collaboration, and is a problem for US astronomers, who need to start their project quickly.

The US National Optical Astronomy Observatories (NOAO) plan to build two 8-metre optical-infrared telescopes: one for the Northern Hemisphere at Mauna Kea, in Hawaii, and one for the Southern Hemisphere at Cerro Pachon in Chile. The National Science Foundation (NSF) can provide only half of the estimated cost

that UK and Spanish astronomers will be able to use ESO's Very Large Telescope (VLT), due to start observation in 2000, provided the arrangement is reciprocal and an adequate system of peer review for one another's projects is worked out. Neither Britain nor Spain currently participates in ESO. The VLT will be a revolutionary instrument, comprising four 8-metre telescopes that can be used independently, or in unison as the equivalent of a 16-metre telescope.

SERC, which has about £20 million to invest, has now deferred its decision until December, after the financial and contractual elements of each project have been scrutinized further. The trend towards closer relations between the United Kingdom and other European countries, both in science and economically, may influence the decision. European collaboration is increasingly seen as being essential to maintain competitiveness with the United States and Japan in 'big science' projects such as space science and particle physics. Moves towards stabilizing exchange rates between Euro-

pean Communities countries may also favour the Spanish option. In 1987, SERC's finances were badly shaken when the UK subscription to CERN, the European particle physics centre, increased as a result of the falling value of the pound.

US astronomers now find themselves in a difficult position. Sidney Wolff, director of NOAO, says that the US project has to get started as soon as NSF money becomes available, to avoid cost overruns. Important decisions on optical design and staff appointments must be made before December, she says. By the time SERC reconsiders, the potential UK input into the NOAO project will have diminished, making it a less attractive proposition for the United Kingdom. In the meantime, British astronomers will continue to refine the design of the La Palma telescope. The delay should be less of a problem for the Spanish, who have yet to finalize their own financial arrangements.

The British indecision may also undermine Canadian support for the NOAO collaboration, although Wolff is optimistic that this will not be the case. She says that NOAO may consider other international partners, while SERC makes up its mind.

Peter Aldhous

EUROPEAN SCIENCE

UK science is sold short

London

THE Treasury's system for handling UK contributions to the European Communities (EC)'s Framework research and development programme compounds the under-funding of British science, according to a report from the House of Lords EC committee, published this week.

Under EC law, member states' contributions cannot be met in full simply by diverting money from domestic spending: there must be some 'additionality' to the EC budget. But the Treasury allows only 30–35 per cent of the UK contribution to Framework as truly additional spending, which the Lords committee says "is clearly far too low". British scientists are successful in winning EC grants, recouping the UK contribution. But Treasury policy means that for every pound of EC research money gained in one year, up to 70 pence is clawed back from the following year's domestic spending on research, by a secretive process called 'attribution' against government departments' budgets.

Attribution has worried the UK research councils, who fear their funds from the Department of Education and Science may diminish as EC spending on basic scientific increases (see *Nature* 345, 376; 31 May 1990). The report charges that the Treasury has ignored the relatively small UK input in shaping EC research policy, as one member state amongst twelve. Many projects funded by

the EC do not coincide with UK priorities.

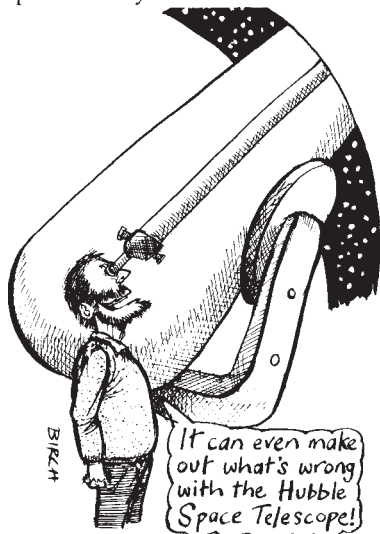
As EC research spending increases, and attribution eats into the domestic budget, "the [UK] science community's ability to set priorities for projects could steadily diminish", the committee warns.

The report says that the Treasury must be more open about the attribution process and suggests that monitoring by Parliament is needed. The Treasury should also study how other EC states manage their contributions — Treasury officials were unable to explain the arrangements made elsewhere in the EC when asked to do so by Lord Shepherd, chairman of the committee.

The committee's inquiry into EC research and development was prompted by the European Commission's proposal for a new Framework programme, to run from 1990 to 1994, overlapping with the existing programme which finishes in 1992. The peers are satisfied with the new programme's budget, and the balance of funds between different areas of research. But the report says that the Commission needs to take more account of the views of the scientific community in individual member states. This could be achieved by appointing representatives from bodies such as the UK research councils to the EC committees that advise the Commission on science and technology.

Peter Aldhous

■ New science minister, page 308.



of about \$170 million, so US astronomers have been seeking foreign collaborators in Britain and Canada.

But Britain has been torn between the US collaboration and a plan to build a Northern Hemisphere 8-metre telescope in collaboration with Spain at La Palma in the Canary Islands. Here, Britain would take the lead in the instrument's construction, basing its design on the smaller William Herschel telescope, also at La Palma.

Britain's main interest is in observing northern skies. A collaboration with Spain would give more observing hours in the Northern Hemisphere, probably at a slightly lower cost, but this must be offset against the better conditions for infrared observation in Hawaii. Both projects aim to have their telescopes ready for use by the late 1990s.

The Spanish collaboration would also allow British astronomers observing time in the Southern Hemisphere. Harry van der Laan, director general of the European Southern Observatory (ESO), says

Valuable cancer registry

Munich

GERMAN reunification may bring an unexpected boon to the science of epidemiology in West Germany. Unlike West Germany, where strict laws to protect privacy have prevented the accumulation of national records on the incidence of cancer, East Germany has detailed records of all cancer cases since 1953.

West German doctors attending the 'first German medical congress' last month in Dresden spoke out for maintaining the East German registry and for trying to establish more cancer registries on a regional basis in West Germany. Georg Dhom, a pathologist in Homburg/Saar, said that a senior health ministry official had indicated at a meeting last month that the federal government would consider establishing a 'national framework law' to allow the collection of data from cancer patients. Dhom is a member of a health ministry working group concerned with cancer epidemiology.

But as health policy is decided in West Germany by the *Länder* (states), the creation of a framework law could only be a first step. Such a law might encourage

the establishment of regional cancer registries similar to those in Britain. West Germany has only two such registries now, in Saarland and in Hamburg.

Even though mandatory registration of cancer cases is forbidden by strict privacy laws in West Germany, Dhom said that the Saarland cancer registry has functioned well with voluntary participation by doctors. Doctors submit data together with the identification of cancer patients to the registry, which removes the names to protect the patients' privacy.

But he criticized as "expensive and technically difficult" the method chosen for a new registry in Baden-Württemberg. There, data are made anonymous before being reported to the central registry; Dhom predicted that double entries will become a major problem. The main reason this method was adopted, he said, was to please data protection activists who fear meddling by the state. But Dhom said that cancer patients in West Germany are in favour of more cancer registries and that opponents have succeeded in preventing their creation by using "very irrational" arguments.

Steven Dickman

NAZI VICTIMS

Money, money, money

Munich

THE West German science council Wissenschaftsrat on 11 July called for massive aid for teaching and research in what is now East Germany in order to raise standards and make its universities more attractive to students and professors. At least DM6,500 million (about \$4,000 million) will be needed by 1995, exclusive of running costs, said the council.

Good research and advanced study should be moved back from the East German Academy of Sciences to "diverse and properly functioning" East German universities, according to the council.

One way to do this, it suggested, is to remove from the academy the right of granting advanced degrees and teaching qualifications (*Habilitation*).

The council urged East German institutions and Western reviewers to resist the pressure simply to impose the West German system on East Germany. Instead, West Germany should take the opportunity to identify its own weaknesses and correct them.

The council also supported an increase of at least 25 per cent in the budgets of the Alexander von Humboldt Foundation and the German Academic Exchange Service (DAAD) to promote the international exchange of senior and junior researchers as well as students in a united Germany. **S.D.**

Graves desecrated

Munich

UNKNOWN persons on 15 July desecrated the graves where body parts and tissue samples taken from the corpses of Nazi victims had been buried 11 days earlier. The samples had been in the collections of the University of Tübingen and were used for research and teaching purposes until a scandal in 1989 prompted their removal and eventual burial.

The criminals spray-painted swastikas onto the gravestones in the Tübingen city cemetery and hacked to pieces the stone that commemorated the victims.

The University of Tübingen held a commemorative ceremony on 8 July in honour of some 400 people whose remains were discovered in the collections of the Anatomy Institute. At the recommendation of an external committee, the university removed from the collections the remains of anyone who was known or even suspected to have been killed by the Nazi regime.

The destroyed stone was carved with a motto written by University of Tübingen pathologist Jürgen Peiffer: "Abducted, oppressed, violated./Victims of despotism or blinded justice./People did not find their rest until they arrived here./From their corpses even,/was demanded a service,/by a science that did not respect human values and dignity. /May this stone serve as a warning to the living." **S.D.**

Data released at last

Washington

THE controversy over whether exposure to low-level radiation is linked to an increase in cancer deaths looks set to be refuelled after the US Department of Energy (DOE) last week finally released data on the radiation exposure and mortality rates of workers at nuclear weapons plants. The data are being released to the Three Mile Island Public Health Group which disputes the the official DOE opinion that low levels of radiation are safe because at worst they are linked only to one very rare form of cancer. DOE officials have until now been reticent to release their data, claiming that information on workers' health is confidential.

Success for TMI in a three-year lawsuit came only after pressure from Congress and the recommendation of a DOE panel of enquiry that public access be given to workers' health statistics.

This first installment of newly released data includes records of the radiation exposure and mortality rates of 44,000 people who worked at the DOE's Hanford Facility in Washington between the 1940s and 1981. Hanford is the DOE's oldest nuclear weapons manufacturing plant.

TMI will eventually have access to data on 200,000 workers, one-third of all of DOE's personnel. But the terms of the legal agreement between TMI and DOE prevent TMI from releasing this information outside their research team.

With the new data, the 83-year-old TMI epidemiologist Alice Stewart hopes to update her earlier controversial studies with the Hanford population of workers. Her work was stopped in 1977 when her supervisor, Thomas Mancuso, lost his contract with DOE. That decision was in itself the subject of a congressional hearing.

Mancuso and his co-workers had published a study suggesting that increases in many kinds of common cancer could be seen at levels of radiation exposure that DOE considered safe.

Rival epidemiologists, including those under contract with the DOE and independent groups in British and Canada, dispute this finding. According to DOE contract researcher Ethel Gilbert of Battelle Pacific Northwest Laboratory, they have found only suggestions of a correlation between low-level radiation exposure and the occurrence of multiple myeloma, a very rare cancer, in the DOE worker population. Data collected in Japan from studies of A-Bomb survivors do not support Stewart and Mancuso's conclusions.

DOE officials are currently considering how they will make data on all their workers available to the public. **Robin Eisner**

Fujitsu's long march

London & Tokyo

FUJITSU, Japan's largest computer maker, is to bid for a majority shareholding in ICL, the only British company still manufacturing mainframe computers. News of the deal, leaked to the press two weeks ahead of the planned announcement, has sent a shockwave through the computer industry as it is seen as the most daring of a series of moves by Japanese companies determined to build global competitors to the US giant, IBM.

If successful, the bid will take Fujitsu from the number four position, in terms of global revenues, to the number two position, leapfrogging NEC of Japan and Digital Equipment Corporation of the United States. But Fujitsu and ICL's combined revenues for 1989 were still less than a quarter that of IBM.

Fujitsu is expected to bid for at least a 50 per cent holding in ICL at the end of this month. The Japanese company would gain a profitable subsidiary (ICL had pre-tax profits of £145.7 million last year) and easy access to the computer market in the European Communities (EC), when trading restrictions between the EC partners are abolished in 1992. Some link up between the two companies had been expected, since Fujitsu already supplies ICL with semiconductors for its machines. But few predicted that STC, ICL's parent company, would be prepared to give up a majority stake.

Fujitsu's takeover bid is just one of several moves by Japanese computer manufacturers intended to form alliances against IBM with European and US companies. European companies are not themselves seen as serious competitors; with the backing of the Ministry of International Trade and Industry (MITI), Japanese companies are concentrating single-mindedly on overtaking IBM.

Apart from its ICL link, Fujitsu has a major shareholding (43.6 per cent) and cooperative marketing agreement with the US computer maker Amdahl, which uses Fujitsu technology in its computers. The Japanese company also has an agreement with Siemens of West Germany, whereby Siemens sells Fujitsu computers with Siemens labels on them.

Hitachi and NEC have gone into similar licensing agreements and joint ventures in Europe, producing a marketing network for Japanese companies that in total matches IBM's in scale.

UK Opposition Labour party technology spokesman Lewis Moonie says he is "disappointed that the only remaining British mainframe manufacturer has to pass into foreign hands". He blames the lack of a coherent government strategy for information technology for the current state of the British computer industry, and

is also concerned about the takeover of the UK software company Hoskyns by the French group Cap Gemini Sogeti, announced last week.

But Martin Campbell-Kelly, who lectures in the history of the computer industry at the University of Warwick, does not view the ICL takeover as the death knell for the British computer industry. Rather, he says, the industry has moved on from the manufacture of hardware to the production of integrated computer systems assembled from hardware manufactured elsewhere.

Campbell-Kelly says that two important

OCEAN RESEARCH

Japan dreams deep dreams

Tokyo

JAPAN'S ambitions to have the biggest and the best of everything appear to have touched the Ocean Development Division of the Science and Technology Agency, which last week sent in a request for funds to begin work on the design of the world's largest ocean drilling ship.

The initial request is for just ¥97 million (\$620,000). But the design is to be for a ship of 18,000 tons (gross) — more than twice the size of the US ship *JOIDES Resolution* which is used in the International Ocean Drilling Program. Yoshiro Miki, director of the Ocean Development Division, says the ship would be equipped with all the most sophisticated Japanese instruments, would cost about ¥30,000–¥40,000 million (\$200–\$270 million) and would be ready before the end of the decade.

The International Ocean Drilling Program is scheduled to end in 1993. But it may be extended by another five years or so, in which case the Science and Technology Agency will discuss how to share the drilling activities of the two ships with the countries involved in the programme.

Somewhat surprisingly, the Ministry of Education, Science and Culture, which at present supports Japan's participation in International Ocean Drilling Program, has not expressed any opposition to the Science and Technology Agency's plans. The two organizations often fight for territory in basic research but Miki says ocean research is one area in which they have learned to collaborate.

The only thing that might thwart the agency plans to build the drilling ship is the Science and Technology Agency's commitments to space research. The agency's participation in the US space station project will drain its resources over the next decade. But Miki and other agency officials are determined to try to maintain a well-balanced budget for all

questions surrounding the ICL deal remain unanswered. The first is whether Fujitsu will discontinue ICL's mainframe range to concentrate on marketing their own products. The second is what will happen to ICL's research and development base once the company passes into Japanese hands.

Some UK observers predict a diminution of research, but Japanese electronic companies typically re-direct large percentages of their profits into research and development, suggesting that Fujitsu will make full use of ICL's research capabilities. Several Japanese computer manufacturers have already set up research units in Europe and the United States.

Peter Aldhous & David Swinbanks

areas of scientific research.

The Ocean Development Division has other new projects, besides the drilling ship, which it hopes to cover with a total 24 per cent increase in its budget for next year. The budget request, for ¥12,000 million (\$80 million), will almost certainly be whittled down by the Ministry of Finance over the coming weeks but is in line with recommendations published by the Council for Ocean Development of the Prime Minister's Office in May and a call for increased research on Earth science and technology issued last month by Japan's highest science policy-making body, the Council for Science and Technology. Miki says he is confident that the new projects will be funded.

Biggest among them is the DEEP STAR (Deep-sea Environmental Exploration Program-Science and Technology for Advanced Research) project, which aims at growing deep-sea microorganisms in the laboratory at pressures up to 650 atmospheres (the pressure at a depth of about 6,500 metres in the ocean). The project began this financial year with a budget of ¥790 million (\$5.2 million) to build equipment for collecting microorganisms from the sea floor. A total investment of about ¥4,500 million (\$30 million) will be required over three years to complete facilities on land, Miki says.

Koki Horikoshi of the Institute of Physical and Chemical Research (RIKEN), who won international recognition for his 'Superbug' project (see *Nature* 310, 172; 1984), will head the DEEP STAR project at the Japan Marine Science and Technology Center (JAMSTEC).

The budget request also includes several hundred million yen to study ocean circulation and climate change near the North Pole in collaboration with the United States and West Germany.

David Swinbanks

Battle lines are drawn

Washington

UNDER pressure from both animal welfare groups and scientists, two groups with widely divergent views of how the government should regulate the use of research animals, the US Department of Agriculture (USDA) last week opted for a compromise; it issued comprehensive new rules on the care of guinea-pigs, hamsters and rabbits, but inserted language to ensure that few laboratories will be forced to buy new cages or make other expensive changes to conform.

The new rules are the first of two controversial sets of regulations to make it all the way through the regulatory process and to be published in the *Federal Register*. Both have been the subject of a five-year struggle between the agency, Congress and activists. As such, the battle over them has been mostly one of principle; the guinea-pig and rabbit regulations are seen as a precedent-setter for the still-unresolved and much more contentious regulations for non-human primates and dogs (see *Nature* 345, 755; 28 June 1990).

At issue is the concept of "performance-based" versus "engineering" regulations. Scientists argue that they should be given some leeway to design animal caging that does not exactly conform to some minimum size standard as long as they can show that the animals are healthy and happy. Animal welfare lobbyists, on the other hand, say such performance-based regulations are unenforceable, and that anything other than strict size standards are no standards at all. The Animal Legal Defense Fund, which has sued USDA to release the primate and dogs rules, is considering a lawsuit over the guinea-pig and rabbit regulations as well.

Although the new guinea-pig and rabbit regulations lay out strict cage-size mini-

mums, they are "grandfathered" — they apply only to new caging bought after the rules take effect next month. More importantly, the rules allow an exemption for "innovative enclosures that do not precisely meet the space requirements". What that means is that good cages are not necessarily standard boxes, says John Miller who, as director of the animal welfare office of the National Institutes of Health (NIH), helped to write the new regulations. Because, for example, hamsters prefer walls to open spaces, if one were to design a hamster cage that was a series of interconnected tubes, which maximize the wall area but may not quite meet the minimum floor space requirement, the enclosure would still be perfectly acceptable, Miller says.

"That's precisely why the 'innovative enclosures' clause was inserted — to give the regulations that performance flavour". Miller explains. But he stresses that the clause does not offer *carte blanche* for scientists to ignore the size standards. "Smaller than minimum does not constitute innovative", he says. At any rate, because the new regulations put into law the NIH guidelines that have been in place since 1972, almost all NIH-funded animal laboratories are already in conformance, Miller says.

The still-unfinished rules for primates and dogs are now likely to also include an similar "innovative enclosure" clause, according to Miller. Since the White House rejected the USDA's previously proposed engineering-based standards earlier this year (see *Nature* 344, 804; 26 April 1990), the agency has retreated to lick its wounds and rewrite the regulations in performance-based language. It intends to resubmit them for review and public comment this fall.

G. Christopher Anderson

US SPACE PROGRAMME

Stone voyages to JPL

Washington

VOYAGER 2, the phenomenally successful interplanetary probe whose flyby of Neptune last year provided a much-needed boost to the National Aeronautics and Space Administration (NASA) space programme, has left yet another success in its wake. Edward Stone, Voyager's scientific director, has been selected as the next head of NASA's top planetary and robotics facility, the Jet Propulsion Laboratory (JPL). Stone will replace Lew Allen, who is retiring in December.

Stone will be taking over a laboratory that has seen much of its original robotics mission eroded by an increasing NASA emphasis towards manned space exploration.

He says that broadening JPL's base beyond planetary science — a process already begun by Allen — will remain a top priority. Likely areas of growth include work on parallel-processor supercomputer, radiation-hardened electronics, robotics and instrument-building for other NASA laboratories. Further changes at JPL — including a possible increased emphasis on missions focusing on cosmic particles and fields, Stone's scientific specialty — may be some time off.

JPL's development over the next two years has already been set and any new direction at the laboratory will have to wait, Stone says.

G. Christopher Anderson

Research still in favour

Paris

THE French government has once again made education and research top priorities in its annual budget. Although ministers have been told only the global figures they can expect — it is up to them to propose how the money should be spent — education is set to receive 9 per cent more than last year and research and technology an increase of 7.5 per cent. Inflation in France is currently 2.5 per cent.

The national education budget, which includes university research, but not other state research money, will increase by FF20,000 million (\$3,610 million) to FF250,000 million. The increase will be swallowed up by a major new construction drive within higher education and ambitions to create thousands of new posts in all sectors of education.

Unlike most of its neighbours, France will not decrease its defence budget next year. Jean-Pierre Chevènement, the defence minister, has apparently succeeded in fighting off proposals to cut his budget and has even won a 3 per cent increase, just ahead of inflation. But greener issues are still close to President François Mitterrand's heart: the environment ministry will see its budget up by 15 per cent in 1991.

Ministers and civil servants now have the summer to prepare detailed budgets in time for a cabinet meeting in September.

Peter Coles

UK RESHUFFLE

New minister for old

London

ROBERT Jackson, UK minister for science and higher education, has been moved from the Department of Education and Science (DES), as part of a reshuffle of middle-ranking ministers designed to improve the Conservative government's image and performance in the run up to the next general election. Jackson had been widely criticized for his piloting through parliament of a bill introducing loans for students, and the failure to convince the major banks that they should administer the scheme. More recently, he was attacked by colleagues after joking at London's Heathrow airport that his ministerial box contained a bomb.

Michael Fallon, newly appointed to the DES, is expected to take over Jackson's responsibilities. The British scientific community will hope that Fallon's appointment introduces a more sympathetic attitude from the DES towards the need for increased funding and improved career prospects in UK science. Jackson has maintained that the UK 'brain drain' is a myth, and was labelled "the rottweiler of academia" at recent press conference by the pressure group British Scientists Abroad.

Peter Aldhous

HUMAN GENOME PROJECT

The honeymoon is over

Washington

AFTER an infancy of accolades from politicians and scientists alike, the Human Genome Project is finding that growing up is hard. The House of Representatives Appropriations Committee last week cut the project's 1991 funding to \$66 million — nearly \$34 million less than the president's request, which was itself \$24 million less than genome project officials had originally asked for. Overall, the House figure is about one-half of what genome project planners had hoped for next year and reflects a substantial erosion of confidence in the project among legislators.

House Appropriations Committee staff say they are concerned that the project, which is orientated toward centres rather than individual grants, would contribute to the trend at the National Institutes of Health (NIH) away from support of single investigators. They also say that project officials have made a poor case for their budget request, by providing detailed numbers only when asked and by lifting much of the request from the recommendations of a 1988 National Academy of Sciences report.

Staff say that although a letter-writing campaign by scientists opposing the project was not a major factor in the House decision, appeals from other researchers in support of more individual investigator grants were persuasive.

NIH genome centre director James Watson says the House has missed the point. "I think they've misunderstood the role of centres in the project", he says. Much of the sequencing and mapping of the project is routine work best suited to a small number of well equipped laboratories. Although fundamental research is often well suited to small laboratories and single researchers, the genome project requires something closer to factories. "Congress needs to make the distinction between exploratory research and getting the job done", Watson says.

Legislators on the Senate Appropriations Committee side with Watson on the initiative. But although they say they will try to vote the president's full \$108 million request, the vagaries of the budget process have left the committee with nearly \$2,300 million less for NIH as a whole, and it may be forced to make cuts as well.

Once the Senate has arrived at a figure, the two arms of Congress will negotiate a compromise budget in conference. Short of a catastrophic across-the-board cut to satisfy the Gramm-Rudman deficit-reduction provisions, the genome project is likely to get something between the House and Senate figures and far less than the president's request.

Officials at the genome project's NIH headquarters are not counting on a sub-

stantial reprieve. Three new projects that were about to be announced — a new pilot sequencing programme, a mapping database and a project to locate index markers on the genome — are now in abeyance until the picture clears, says genome office budget officer Erin Burgess. NIH had also planned to start nine new centres at about \$4 million each in 1991; the House cuts could lower that to as little as three centres at \$2–\$2.5 million each.

Funding for the three projects now in limbo was to have amounted to about \$19 million next year — just a little more than the \$18 million that Congress has placed tantalizingly out of reach in a special discretionary fund for the director of the NIH. This fund can be made available to the genome project at the discretion of the NIH director, once a director has been

ABORTION PILL

French pill for Britain?

Paris & London

RU-486, the abortifacient pill, could be marketed in Britain by next year if its manufacturer, the French company Roussel-Uclaf, goes ahead with its decision to apply for a product licence. At present, says the company's head of marketing, Ariel Mouttet, Roussel-Uclaf is making enquiries to ensure that the strict controls surrounding distribution of RU-486 in France can be maintained in Britain. If so, the company will send its application to the Department of Health.

At present, RU-486 is only available in France. Even there, it took state intervention to put the drug back on the market after it was withdrawn following threats to company executives from anti-abortion groups (see *Nature* 340, 6; 1989). Only now, after successful clinical trials in Britain and over two years of use in France, is Roussel-Uclaf preparing its first major attempt to market the drug abroad.

The drug already has some supporters in British medical circles and, according to Mouttet, Kenneth Clarke, the health secretary, has written to encourage Roussel-Uclaf to apply for British registration. Naren Patel, honorary secretary of the Royal College of Obstetricians and Gynaecologists, says that "preliminary reports seem very favourable", although he stresses that he has not yet seen all the data on clinical trials. Patel believes that the drug may be more appropriate for certain terminations than existing techniques. A spokeswoman for the British Medical Association was also supportive. "We do not see any immediate medical problems", she said. But she pointed out that abortions still require the consent of two doctors, under the terms of both the

appointed. There has already been a delay of nearly a year in filling the position, which has caused increasing tension between NIH and Congress. House staff say that they want a new director soon, and they want that director to take a new look at the progress and management of the genome project.

Genome centre officials fear that linking the genome centre's fortunes to the arrival of a new NIH director places the project's growth in jeopardy. "We're not feeling very positive about the money. We're hoping the Senate will say you can't hold the genome project hostage for a new director", says Burgess.

The Department of Energy (DOE), which runs about one-third of the project, has so far done much better in the appropriations sweepstakes. DOE funding from the House is \$46 million, just what the president requested.

G. Christopher Anderson

old Abortion Act and the Human Fertilization and Embryology Bill, currently before parliament.

Roussel-Uclaf is also keen to dispel any ideas that RU-486 will be available over the counter as a 'morning after' pill or for 'do-it-yourself' abortions. In France the drug is available only within approved clinics and hospitals and must be taken in the presence of a doctor, in association with synthetic prostaglandins.

Mouttet explained that the company will not proceed with its application unless tight distribution controls are respected. In France the drug is treated in the same way as are opiates and controlled narcotics. At pharmacies it must be kept in a locked cupboard and a complex system of numbered order books, consent forms and sticky labels ensures that every box of the drug can be accounted for.

An estimated 40,000 women have now aborted using RU-486 and Roussel-Uclaf has full records on 20,000 of them. A study of the first 2,000 cases has been published and a report on a further 10,000 women is available within Roussel-Uclaf. Until March this year, the drug was issued free and, says Mouttet, orders were running at "about 4,000 boxes per month". Now there is a charge, demand has dropped to "about 3,000 a month".

If RU-486 is marketed in Britain, the manufacturers will next consider approaching Scandinavian countries. But Mouttet confirmed that the company still has no plans to market RU-486 in the United States or to carry out clinical trials there. If the drug is easily available in Britain, however, a black market for the drug might easily develop in the United States.

Peter Coles & Peter Aldhous

Send Hubble to the Moon

SIR—The proposal of Bahcall in *Nature* of 22 June last year¹ to establish an international institute for astrophysics is an important one. In particular, their suggestion of taking to geosynchronous orbit the 2.5-metre test mirror developed for the Hubble Space Telescope (HST) programme is an interesting possibility.

This is an example of reducing astronomical costs as well as sharing them. In low Earth orbit, the HST must be serviced during the next solar cycle, which may be a costly exercise, possibly compromised by the flight availability of the Space Shuttle. In geosynchronous orbit, on the other hand, an HST instrument would need less servicing, and would provide direct data access (see ref. 2 for a discussion of 90-minute operations). It would have direct command and control, three times the efficiency of observations, and ambient-cooled optics (being further from Earth).

I would like to take this suggestion one step further, and point out that the Hubble test mirror could be taken to the Moon, again as part of a cost-effective strategy. NASA has recently conducted three workshops³⁻⁵ addressing physics and astronomy from the Moon, the first of which contributed to a presidential decision that a return to the Moon would be the next major goal of the US space programme. It is clear that the Moon, as our nearest neighbour, offers distinct advantages for space astronomy over low-Earth orbit and geosynchronous orbit, and over any planetary initiative.

Taking the Hubble test mirror to the Moon, at the onset of the programme, could serve as a focal point for the lunar initiative, placing the programme's emphasis on science and astronomy. In this scenario, the test mirror with modern instrumentation and redesigned actuators for a structural 1/6-gravity environment at a lunar base (rather than zero-gravity in Earth orbit) would be put in place immediately at the landing site on the second or third manned mission to the Moon. (There may be unmanned excursion missions, landing before the manned phase.)

A 10,000-pound Hubble instrument is 4.5 metric tonnes, one of the very payload weights currently used by NASA's Office of Exploration⁶ in its preliminary models for the manned flights there. With the approval of the US Congress, one can easily imagine the Hubble 2.5-metre mirror at a man-tended lunar base within this decade (by the year 2000, one of the schedule dates now in use⁶).

The existing proposals^{3,5,6} on astronomy from the Moon have been described as "lacking vision". Using the Hubble test mirror in this way, that is, putting it in place at the onset (the second lunar landing) at low cost (as an 'off-the-shelf'

instrument) could catch the public imagination and muster the scientific support required for a visionary approach (see H.J. Smith in ref. 5 for discussion). This must not compromise the Hubble programme itself, it must be international in scope, and it must be followed by a more ambitious set of observatories using larger, higher-quality mirrors (since the Hubble test mirror does not meet the needs of space astronomy in the twenty-first century).

The Hubble emplacement, early on, would also serve as a testbed for determining requirements and problems for astronomy from the Moon as the initial outpost evolves into a mature lunar base.

THOMAS L. WILSON

NASA, Johnson Space Flight Center,
Houston, Texas 77058, USA

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Global warming

SIR—Perhaps you have not received any correspondence that presents a positive or optimistic outlook for the changes that are predicted to accompany or be a consequence of global warming? The good news that large areas of Canada and the Soviet Union may become more conducive to agriculture seems largely discounted. More optimistic speculations could centre on how the results of the greenhouse effect might circumvent the burning of fossil fuels for energy. Apart from the argument that we may need to burn less fuel for heating in a warmer world, going further, desert areas could become the 'oil wealth' of the future if they were turned over to massive-scale production of solar-generated electricity. This abundant electricity might be used to make hydrogen for use in internal combustion engines. Or perhaps there could be solar-powered desalination plants to produce fresh water for use in, say, irrigation programmes. Such schemes would require labour, but might appeal to people forced to move if their homes became uninhabitable as a result of climatic change.

Such an optimistic outcome would, of course, require planning, management and co-operation between nations on an unprecedented scale. Such intergovernmental co-operation may seem unlikely, but an unbalanced, gloomy outlook surely

cannot help it to come about. I hope you will encourage the avoidance of doomsaying in such matters.

DAVID GILBERT

Department of Biology,
Open University,
Milton Keynes,
Buckinghamshire MK7 6AA, UK

Australia prize

SIR—Tania Ewing's story on the Australia Prize (*Nature* **344**, 692; 1990) needs correction.

Professor Allen Kerr did not isolate the bacterium responsible for crown gall disease. That was done long ago. He was, however, the first to show that pathogenicity in *Agrobacterium tumefaciens* could be transferred from one strain to another.

This transfer was then shown by the groups associated with Professors Eugene Nester and Jeff Schell to involve the large tumour-inducing plasmid (pTi). This was the beginning of the development of an effective plant transformation system, to which their groups have contributed so many advances.

Ewing errs again in attributing to them the genetic engineering of a benign strain of *Agrobacterium* to ensure continuing effectiveness of the system of biological control of crown gall disease. That was due entirely to Kerr's group.

DAVID CURTIS

Australian National University,
John Curtin School of Medical Research,
GPO Box 334,
Canberra, ACT 2601,
Australia

Crisis in education

SIR—It seems that there is a dearth of applications for postgraduate research awards this year, but there is a widespread reluctance on the part of supervisors to admit how few applications have been received for the awards on offer, as they fear that the poor application rate will discourage funding bodies from continuing their support.

This is a mistake. Rather than concealing the situation, we should be shouting and screaming about the low number of aspiring postgraduates, as it is symptomatic not of problems within individual departments, but of a major crisis within the whole higher education system that must be addressed now. A dearth of postgraduates now will be a constraint on the future development of research in this country.

PETER G. KNIGHT
ANTHONY J. PARSONS

Earth Science Surface Processes Unit,
Department of Geography,
University of Keele,
Keele Staffs ST5 5BG, UK

Can mirrors beat the greenhouse?

People should not personally assume responsibility for the greenhouse effect, which would be impossible, but they should give some thought to the quantities involved.

AMONG the green community, the prospect that the excess greenhouse effect may be a reality, even here and now, has evoked a general wish to be seen to be doing something to assist in its abatement. That is natural enough, even though there are the strongest possible reasons for suspecting that individual virtue will serve only to provide the less virtuous with a licence not to follow suit, which is why the proper regulation of the greenhouse requires an international convention. Meanwhile, a child's guide to some of the steps that individuals might take may also serve as a guide to the underlying simple arithmetic.

At the present average distance between the Sun and the Earth, the solar energy flux normal to the line of sight is roughly $1,370 \text{ W m}^{-2}$, but only some 70 per cent of that reaches the surface of the Earth. (Ultraviolet is mostly absorbed by the ozone layer, which is mostly still there, radio waves are screened out by the ionosphere and so on.) The average sub-zenith solar flux may be taken as 950 W m^{-2} , not very different from 1 kW m^{-2} , but that is only ever nearly valid in the tropics, while half of the surface of the Earth is always turned away from the Sun; the average flux at the surface is therefore one quarter of the sub-zenith flux, say 240 W m^{-2} .

It is important that the mismatch between incoming and outgoing radiation fluxes expected from the accumulation of CO_2 and other greenhouse gases is of the order of 1 W m^{-2} . This, for example, is the degree of what is called in the trade the 'forcing' of the Earth's climate system caused by the accumulation of CO_2 and, to a lesser extent, of methane, between 1850 and 1960 — then the only greenhouse gases worth talking of. (CFCs have become a serious contributor to the problem only since that time.) People bent on personal steps to ameliorate the excess greenhouse effect must keep that figure in mind. What can they do?

First, perhaps, they should consider what might be done with simple mirrors. If one happened to live in the tropics, one might hope that a square metre of silvered glass placed in one's back garden would reflect back to space a substantial fraction of the incident flux of energy — perhaps not the whole of 1 kW , but roughly a quarter of it. But even that is not quite true. When the mirror surface is away from the sub-zenith point, the absorption

and scattering of energy on the longer pathways into and out of the atmosphere will be greater. Perhaps the most one can hope for is that an eighth of 1 kW m^{-2} will on the average be turned away.

Naturally it is worse when, like most people, one lives outside the tropics. Then there is an extra factor of $\cos \alpha$ (where α is latitude) to be taken account of, as well as the extra absorption and scattering in the atmosphere. At latitude 60° , that means a further factor of two on account of latitude, and yet another factor of two on account of extra path length through the atmosphere. If the albedo of the part of the surface on which the silvered mirror is placed is that of a perfectly absorbing black body (which cannot be exactly true), a single square metre of silvered surface will turn back only an average of roughly 30 W. Carpeting a few per cent of the Earth's surface with mirrors would be necessary to counteract the greenhouse forcing so far (which emphasizes why snow cover and sea ice are important in climate models). But an area smaller by a factor of about four would be required in the tropics.

Absolute quantities are important at this stage. In round numbers, the surface area of the Earth (two-thirds of which is water) is $5 \times 10^{14} \text{ m}^2$, which means that the greenhouse forcing of the past 150 years is the equivalent of $5 \times 10^5 \text{ GW}$ of energy — not very different from the world's present production of electricity.

Even if one takes the view that one's personal responsibility is limited to one's fair share, it would still be necessary, if repairing past damage is the goal, to be personally responsible for returning to space the equivalent of 100 kW of solar energy, requiring an investment in some $3,000 \text{ m}^2$ of mirror surface outside the tropics or in something like 750 m^2 in the Sahara. If, on the other hand, one took the view that it would suffice to neutralize by mirrors one's incremental contribution to the excess greenhouse effect, then because the next fifty years are expected to be about as damaging as the past 150 it would suffice to lay 150 m^2 of one's non-tropical back garden with mirror surface every year, or to arrange that an agent acting on one's behalf in the Sahara similarly treats 20 m^2 or so of desert surface every year.

This argument is a *reductio ad absurdum*, and is meant to be. (Most people do not have back gardens that big, in any

case.) But the orders of magnitude are important. The calculated imbalance between the incoming and the outgoing radiation is of the order of a few kW per person now alive. It must surely be relevant that most of the 5×10^9 of us do not have the opportunity to consume that much electricity.

The opportunity for a technical fix arises at this point. The green community naturally abhors technical fixes, often on the grounds that they are palliatives rather than permanent 'solutions', but it should not go unremarked that the most economical solution of the greenhouse problem by means of mirrors must be that light-weight mirrors, no doubt made of aluminium foil, should be installed in the line of sight between here and the Sun.

No doubt there would be countless opportunities for endlessly more sophisticated calculations of the effects of radiation pressure on such structures. There have already been several calculations in the past of how such devices might be used to channel solar energy towards collecting stations on the surface of the Earth. Faced with the contemporary version of the problem — how best to get rid of solar energy — the space research community would no doubt be able to rise to the challenge.

That is why the true essence of the greenhouse problem is not the question of whether the concentration of CO_2 in the atmosphere is increasing (it is), or that of the degree to which CFCs are more important as greenhouse gases than CO_2 , but that of how the accumulation of these materials in the atmosphere will affect the average temperature on the surface of the Earth and then, more subtly, the climate.

So the responsibility falls upon the shoulders of the modellers. What is the coefficient relating CO_2 concentration to the average temperature on the surface of the Earth? The second is evidently a function of the first, and the function is to a first approximation linear, at least when the processes are slow enough, so there must be a number relating one to another. The role of that number is not to tell us whether or not we should be concerned, but to help us to decide whether we should stay awake at nights. But such a challenge is hopelessly premature: the modellers are still at a loss to know what they should do about real clouds as opposed to the 'average cloudiness'.

John Maddox

Dagmar Ringe and Gregory A. Petsko

Cutting *et al.*² examined the DNA sequences from exons 9, 10 and 11 and exons 20, 21 and 22 in 38 patients. These

Given the imprecision of the method used, it is pertinent to ask whether it should be trusted, much less published. The authors admit that the model is likely to be wrong in detail and may even be

Hyde *et al.* argue convincingly that the protein is a transporter but not a chloride channel, but they do not suggest what it

transports. It is possible, though, that a function can be proposed based on structural considerations: given the connection between symptoms and the defective regulation of chloride secretion, the protein might transport a molecule that regulates the chloride channel. Mutations could affect chloride secretion by altering the amount of regulator available.

But what is the identity of this molecule? The closest homologues to the cystic fibrosis gene product are the yeast STE6 gene product and the MDR (multi-drug resistance) proteins. Conjugation of hydrophobic drugs to glutathione could be important for *in vivo* export by MDR (ref. 12). Such conjugation has not been observed but the physiological substrates for the MDR proteins have not yet been identified. The substrate for STE6, however, is known to be the farnesyl-derivative of the 12-residue α -mating factor peptide^{13,14}. The farnesyl moiety is attached by means of a thioether linkage to the C-terminal cysteine of the peptide¹⁵. From this evidence we can build up a picture of what the substrate molecule for the cystic fibrosis gene product might be like. It would conjugate through a thioether linkage to a glutathione, cysteine or similar residue; it would have a large, hydrophobic organic moiety; and it would have a demonstrable effect on chloride conductance. The products of the arachidonic acid pathway, leukotrienes and prostaglandins, meet these criteria.

Leukotriene LTC₄ is known to stimulate mucous and chloride secretion, and has a structure very reminiscent of the farnesylated cysteine of the yeast STE6 substrate. Prostaglandin D₂ conjugates with glutathione and cysteine in the presence and absence of the enzyme glutathione S-transferase¹⁶. Prostaglandin D₂ produced the largest change in chloride secretion in experiments with various arachidonic acid metabolites on excised

dog tracheal cells¹⁷. The function *in vivo* of both LTC₄ and PDG2 is unclear, but these considerations suggest that one of these molecules (or a similar compound) is the regulator for the epithelial chloride channel and the substrate for the cystic fibrosis gene product. Indeed, an ATP-dependent transporter that pumps glutathione conjugates, including LTC₄, from various tissues has already been identified¹⁸. Inhibition studies imply that this molecule is not identical with the

EVOLUTION

Life and times of the guppy

Brian Charlesworth

THE life history of a species or population is described by such variables as the age of first reproduction, and the mortality rate and reproductive success of individuals as functions of age. Life histories vary greatly between taxonomic groups: for example life spans in eutherian mammals range from a few months in small insectivores to 80 years or more in humans¹. The age of reproductive maturity and number of offspring per litter show a similar diversity². A substantial body of theory has grown up that endeavours to make predictions about the ways in which natural selection moulds life history, and to explain the diversity of life-history patterns^{3,4}. A new study on page 357 of this issue⁵ puts the standard explanation of life-history differences between natural populations to the test. It is the first case in which life-history differences observed in nature have been reproduced in the laboratory.

Life-history variation between populations of the guppy (*Poecilia reticulata*) are thought to reflect differences in the main species of local predators. In some places, predators take large, adult guppies, but in others the juveniles bear the brunt. Guppies from the former class of population mature earlier, and females allocate a greater proportion of their body mass to the reproductive system and have a larger number of offspring per brood compared with guppies from the second type of population⁵. These differences accord with the predictions of the standard 'reproductive effort' model of life-history evolution^{3,4,6}. In species with multiple episodes of reproduction, this model assumes that, for individuals of a given age, the allocation of resources between reproduction and other activities (such as survival to the next time of reproduction) may vary.

Other things being equal, the greater the reproductive effort of an individual (that is, the larger the proportion of resources allocated to reproduction), the lower its chance of survival. The life history that is evolutionarily optimal can

MDR protein¹⁹, but it could still belong to the family that contains MDR, STE6 and the CF gene product. Sequence information about this pump will establish whether the family has yet another member. □

Dagmar Ringe and Gregory A. Petsko are in the Departments of Biochemistry and Chemistry and the Rosentiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02254, USA.

be calculated for this model^{3,4,6}. One prediction is that the level of reproductive effort favoured by natural selection should be strongly influenced by the relative amounts of mortality of adults and juveniles, imposed by external factors such as predation. Relatively high adult mortality favours the evolution of a life history in which there is high reproductive effort. The intuitive reason for this is clear: if the chance of getting through to the next opportunity for reproduction is inherently low, one might as well reproduce now, rather than get killed off. In addition, relatively high mortality tends to select for genes that increase survival and reproduction early rather than late in life³.

Differences between taxa can often be interpreted in terms of this demographic influence on life-history evolution³. For example, there is a positive correlation among bird species between survival rates in the wild and the age of reproductive maturity, and a negative correlation between clutch size and survival⁷. But comparative studies are always open to the objection that factors other than those imagined in one's favourite theory are responsible for the correlations observed. This objection is met in various studies in which artificial changes in the demography of laboratory populations produce evolutionary responses of the kind predicted by life-history theory^{8,9}. Of course, such studies merely show that genetic variability of the kind postulated in the models can be exploited by selection: they do not prove that the invoked selective agents are actually responsible for producing the observed differences.

Reznick *et al.*⁵ have used the guppy system in a neat study that combines both comparative and experimental approaches. In 1976, they transplanted a population of 200 individuals from a river in Trinidad with high predation of adults to a tributary where guppies were previously absent, and the local predator was a species that ate juveniles rather than adults. The transplanted population has

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flourished, and has been monitored at intervals between 1978 and 1988 — a total of 11 years (30–60 generations) in the new environment. Females in the new site now tend to produce smaller brood sizes and have lower reproductive effort than those from the original locality. Laboratory-reared guppies from both localities showed a broadly similar pattern of differences, indicating that there has been a genuine evolutionary response to the changed conditions. An interesting aspect of the laboratory data is that only reproductive effort early in life seems to have been reduced in the introduction site. This is similar to the results obtained for *Drosophila* populations selected for longevity⁸, and presumably reflects the greater selective impact of changes in early life that is at the core of evolutionary explanations of ageing^{3,10}. It would be interesting to know whether age-specific

mortality of the guppies under laboratory conditions has also been modified. □

Brian Charlesworth is in the Department of Ecology and Evolution, The University of Chicago, 1103 East 57th Street, Chicago, Illinois 60637, USA.

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CHEMISTRY

A new twist to self-assembly

Edwin C. Constable

ATTEMPTS by synthetic chemists to imitate the natural world have flourished in the past decade, which has seen the design of artificial enzymes, photosynthetic species and self-assembling molecules. On page 339 of this issue¹, Lehn *et al.* describe the preparation of a new class of compounds that mimic the double-helical structure of DNA. One of the remarkable features of these compounds is the spontaneous assembly of the double-helical arrays from linear precursors through their interaction with monovalent copper centres. Their structure is a curious 'inside-out' analogue of that of DNA.

Copper(I) has a preference for near-tetrahedral geometry, and the secret of helix-building in the work of Lehn's group lies in the choice of a polynucleating ligand that can bind in a bidentate mode at a sequence of metal centres. Ligands based on oligopyridines prove to be ideal for this purpose, and the tetrahedral geometry at each centre combined with the correct 'spacer' groups between ligands allows the construction of double-

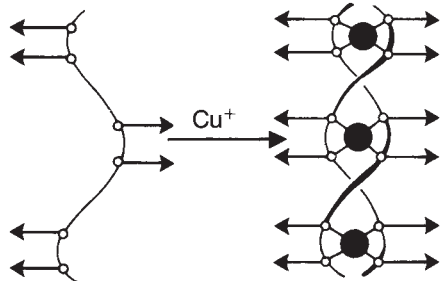


FIG. 1 Lehn's method for the spontaneous assembly of double helices. Key (both figures): nitrogen donor atom, ○; reactive functional group, ●; copper, ●; nucleoside, —→.

helical ligand arrays about a series of metal centres² (Fig. 1).

In their new work, Lehn's group have introduced deoxynucleoside substituents onto the outside of double helices constructed about three or five copper(I) centres. The molecules so constructed show many of the characteristic structural features of nucleic acids, but the overall structural consequences are very different. Lehn's new compounds and DNA all have double-helical geometries, contain deoxynucleosides, are charged and exhibit stacking interactions between approximately coplanar aromatic rings. But in DNA the deoxynucleosides are within the double helix, the stacking is between the deoxynucleosides, and the overall charge is negative; whereas in Lehn's compounds the deoxynucleosides are on the outside of the double helix, the stacking is between the oligopyridines and the overall charge is positive. As Lehn *et al.* point out, the structures of the new molecules are strikingly reminiscent of Linus Pauling's original proposal for the structure of DNA, which had the deoxynucleosides on the outside and the charged phosphate groups facing in.

These interesting new compounds offer opportunities to investigate an entirely new class of DNA-molecule interactions. The extra-helical deoxynucleosides of Lehn's compounds might intercalate or enter into triple-helix hydrogen-bonding interactions with DNA. The metal complexes are positively charged and should show strong electrostatic interactions with the negatively charged surface of DNA and possibly a helix-helix recognition for the major groove. The develop-

ment of double-helical complexes containing other photoactive metal centres might allow the formation of new site-specific nucleases of the type investigated by Barton *et al.*³. Those octahedral transition-metal complexes containing substituted 1,10-phenanthroline ligands exhibit site-specific non-covalent interactions with DNA. If the metal centre is photo- or redox-active, it is possible to couple photochemical or oxidation changes at the metal centre to oxidative cleavage reactions of the sugar-phosphate backbone⁴.

The specific geometrical requirements of metal centres in their coordination compounds has been exploited in a number of other elegant examples of molecular architecture. Sauvage and his group have made extensive use of the tetrahedral copper(I) centre in assembling a series of particularly beautiful molecules. The simplest of these are the catenanes, which may be viewed as two (or more) links from a molecular chain. These are assembled by ring closure of two functionalized bidentate 1,10-phenanthrolines coordinated to a tetrahedral copper(I) centre⁵ (Fig. 2a).

If two of these 1,10-phenanthroline derivatives are linked by a suitable spacer chain, a double-helical complex is assembled in a process similar to that observed by Lehn *et al.* Ring closure in this compound leads not to a catenane but to a coordinated trefoil knot, from which the free ligand may be obtained upon treatment with cyanide⁶ (Fig. 2b). This remarkable synthesis could be achieved only by using metal-ion control over the conformation of the coordinated reactants.

Terdentate ligands may also be used for the spontaneous assembly of double-helical arrays, an example having been reported recently by Williams's group⁷ (Fig. 2c). Once again, copper(I) was used as the metal centre to control the helix formation. Very recently, second-row transition metals have also been shown to direct the spontaneous assembly of double-helical ligand arrays⁸.

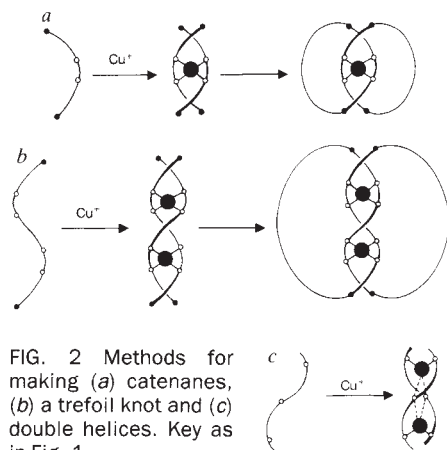


FIG. 2 Methods for making (a) catenanes, (b) a trefoil knot and (c) double helices. Key as in Fig. 1.

Although many of these examples of topological control rely on a metal ion to control the geometry of reactants, it is possible to do this using other intermolecular interactions. Stoddart *et al.*⁹ have recently made use of stacking interactions between electron-rich and electron-poor aromatic systems in a high-yield, metal-free, one-pot synthesis of catenanes (see also ref. 10).

The ability of intermolecular interactions, particularly those involving metal ions, to control the self-assembly of molecular aggregates is an area of intense current interest. The use of such interactions allows very specific control over the conformation and stoichiometry of the products. The methodologies emerging from these studies represent an amalgam of 'organic' and 'inorganic' techniques and expertise, and offer powerful

new approaches to the synthesis of compounds that are of interest for their glorious shapes as well as their potential to exhibit novel and specific interactions with biological molecules. □

Edwin C. Constable is in the Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK.

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TRANSGENIC MICE

Gene expression and hair-loss

Paul E. Bowden

THE regulation of gene expression and the biological consequences of gene manipulation can be studied by the stable incorporation of 'foreign' genes either into somatic cells, or into the germ line of recipient organisms. Transgenic mice result from the successful integration of foreign genes into the germ line; in this way, the introduced genes are subject to the complete developmental programme of the organism. The transgenic approach has recently been used to study the expression and regulation of keratin genes, which encode the structural proteins of hair and skin. In a new report, Powell and Rogers¹ describe the dramatic consequences of expressing a single component of sheep hair (a type II intermediate filament (IF) keratin protein) at high concentrations in transgenic mice. Although expression of this keratin IF gene is tissue specific, its overexpression leads to an imbalance of the normal ordered array of hair structural proteins. The result is a weakened structure, and breaking of the hair shaft (wool fibre) leads to premature loss of hair (see figure). This does not seem to be simply a consequence of expressing a sheep keratin IF gene in a mouse, as mice having a lower level of transgene expression (50 copies or less) suffer no hair-loss. Furthermore, the amino acid sequences of the keratins of sheep wool and mouse hair are very similar².

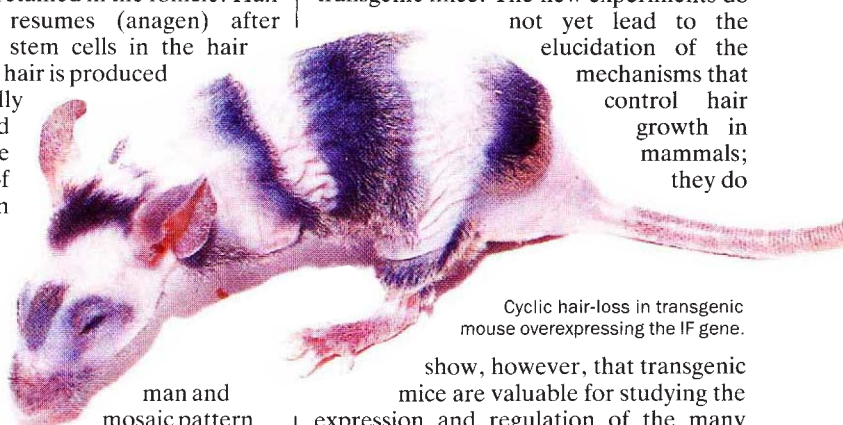
Hair from all mammals possesses a common structural plan and undergoes a cyclic programme of growth and differentiation. The hair follicle is first formed during embryonic development by down-growth and specialization of the epidermal basal cells, which also differentiate to

form the outer layers of the skin³. A complex multicellular structure is formed, which after a period of hair-specific differentiation and growth, produces a hair that protrudes beyond the surface of the skin. The follicle then attains a dormant state (telogen) during which the fully formed hair is retained in the follicle. Hair growth then resumes (anagen) after stimulation of stem cells in the hair bulb and a new hair is produced which eventually displaces the old one. Because the activity of hair follicles in the mouse is synchronized, waves of new hair growth occur in regular cycles⁴, whereas in sheep, a mosaic pattern of active hair follicles interspersed with dormant follicles is seen. Thus, the normal hair cycle, characterized by periods of active growth followed by dormancy, produces a continuous refurbishment of the coat (pelage) without significant loss of hair in any specific area of the skin.

The hair fibre is composed of a cylindrical cortex with a central core (medulla) surrounded by a cuticle. The hair cortical cells contain a regular and distinctive arrangement of filaments (keratin IF proteins) embedded in a matrix of cysteine-rich and glycine-tyrosine-rich filament-associated proteins⁵. Between eight and ten hair-specific keratin IF proteins have been described^{6,7} in addition to the keratin IF proteins expressed in other epithelia^{8,9}. But the synthesis of filament-associated proteins seems to be a late event in hair differentiation and follows the synthesis of keratin IF proteins^{10,11}. So precise regulation of the sequential expression of at least 20 genes is required to construct the filament-matrix array of the hair cortical cell.

Powell and Rogers¹ demonstrate that in transgenic mice with many copies (roughly 250) of the sheep wool keratin IF gene, the overproduction of keratin filaments leads to a reduced synthesis of the sulphur-rich and glycine-tyrosine-rich filament-associated proteins. This disrupts the normal ratio of filament to filament-associated protein, which has profound effects on hair structure and strength. The hairs produced are wavy and tend to break just below the skin surface. Similar findings have been described in several disorders of human hair¹². This leads to the premature loss of hair that the authors observed in their transgenic mice. The new experiments do

not yet lead to the elucidation of the mechanisms that control hair growth in mammals; they do



Cyclic hair-loss in transgenic mouse overexpressing the IF gene.

show, however, that transgenic mice are valuable for studying the expression and regulation of the many structural genes involved in this complex system, and that the biological consequences of manipulating the genome can be difficult to predict. □

Paul E. Bowden is in the Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, UK.

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Stellar birth control

A. P. Whitworth

STAR formation is concentrated in giant molecular clouds, which range in size from 10 to 100 parsecs, and in mass from 10^4 to 10^7 solar masses. The molecular clouds are very fragmented and porous, with most of their mass concentrated in numerous dense clumps. The challenge facing the astrophysicists attending a recent meeting* was to establish the details of this structure, how it is created, and how it evolves. For these are the processes that control the efficiency of star formation in a giant molecular cloud, the initial mass function (the relative proportions of stars formed with different masses) and the initial orbits of binary stars and star clusters — in short, the processes of stellar birth control.

Evidence of the porosity of giant molecular clouds (GMCs) comes from observations of emission lines from ionized carbon, atomic carbon and carbon monoxide in the cloud next to the nebula M17 (J. Stutski, Max-Planck-Institut, Munich). The coincidence of the peaks in the emission lines from these different species implies that far-ultraviolet radiation penetrates deep into the cloud, and thus maintains (by photodissociation and photo-ionization) a distribution of atomic and ionized carbon that is broadly co-incident with the distribution of carbon monoxide. This radiation travels between patches of CO with little attenuation, ensuring that each patch is surrounded by an envelope of the atomic and ionized species.

The sub-structure of GMCs is fractal (E. Falgarone, IRAM Grenoble). In maps of molecular line emission, the isophotes (lines of constant brightness) satisfy the relationship $P \propto A^{0.68}$, where P is the perimeter of the isophote and A is the area enclosed. The amount by which the exponent exceeds $1/2$ (the value expected for isophotes with simple shapes) is a measure of the 'knobbliness' of GMC sub-structure. The fact that this relation holds over a large range of P (from 0.02 to 3,000 parsecs) indicates that the same degree of knobbliness is found on widely different scales. Falgarone's result is remarkable because the fractal dimension is the same as that observed in two-dimensional slices through high-Reynolds-number (well developed) turbulence in the laboratory, and therefore seems to corroborate the theory that GMCs are turbulent.

It is surprising that interstellar turbulence mimics terrestrial turbulence so closely. Terrestrial turbulence is essentially incompressible and not self-

gravitating; turbulent energy is injected continuously on the largest scale and cascades without dissipation through a large inertial range until being dissipated eventually on much smaller scales. Interstellar turbulence, on the other hand, is compressible and self-gravitating; turbulent energy is injected and dissipated stochastically and on a wide range of scales, perhaps even being amplified gravitationally in a cascade to larger scales.

Two sources of turbulent energy in GMCs are ionizing radiation and stellar

of sparsely distributed, predominantly low-mass, star formation (P. Myers, Harvard Center for Astrophysics). The masses of protostellar cores reflect the properties of the clouds within which they form, in the sense that more massive cores (and hence presumably more massive stars) form in more massive clouds and hence in deeper, steeper background gravitational potential wells. If this tendency is universal, it explains why star formation in cooling flows (extended envelopes of very hot, X-ray-emitting gas around elliptical galaxies) produces only low-mass stars, and it suggests that globular clusters had a high proportion of massive stars early in their evolution.

One can speculate that 10–20 million

IMAGE
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REASONS

The Cone Nebula, in the constellation Monoceros, is the prototypical example of a cometary nebula. The bright 'fireball' at its apex is a cloud of ionized gas which casts a shadow in its wake.

winds from associations of massive, luminous OB stars. In the Orion/Ophiuchus star-forming region, the influence of the central OB association is felt out to a distance of 80 parsecs (J. Bally, AT&T Bell Laboratories), as indicated by the systematic orientation of cometary nebulae. These nebulae are so called because of their comet-like shape, characterized by a very dense head and a less dense tail, the whole of which may be enclosed in a sheath of ionized gas. The Cone Nebula (see figure) is prototypical. In Orion/Ophiuchus the heads all point towards the central OB association, suggesting that the nebula boundary is defined by a bow shock and/or ionization front where the collective stellar wind and/or ionizing flux from the central stars encounters the material in the head. The tail is simply the shielded region in its wake.

Cometary nebulae are important sites of star formation. The heads sustain a high formation rate, resulting in populous clusters which include high-mass stars; whereas the tails support only a slow rate

years after its formation the head of a cometary nebula will be dispersed by the ionizing radiation and winds from the massive stars which have formed within it. The tail will probably persist as an isolated filament of the type observed in Taurus (Y. Fukui, Nagoya).

Despite the wealth of observational data on star-forming regions, the connection between observation and star-formation theory remains tenuous. In star-formation theory, there is only one certainty: stars condense out of interstellar gas through the effect of self-gravity. There is also a near-certainty: self-gravity first pulls together the material to form a star-cluster and then from this it forms the individual stars. But as yet, there is no observational confirmation of either the certainty or the near-certainty. We do not see protostars or protocusters contracting under self-gravity. On the large scale, those parts of star-forming regions that reveal themselves to us appear tenuous and filamentary, more akin to the wispy cloud patterns of a summer's sky than to massive bodies bent on collapse.

* *Fragmentation of Molecular Clouds and Star Formation*, IAU Symposium No. 147, Grenoble, 11–15 June 1990.

On the small scale, we see cold, self-gravitating molecular clumps and infrared sources which indicate where stars have formed or are in the final stages of formation; but there is no unique signature of inward motion.

The observations indicate that thermal instabilities are at least as important as self-gravity in generating sub-structure within GMCs. Numerical simulations (J. Chi  ze, CEA Bruy  res) show that when the ambient ultraviolet radiation field is

PALAEOCLIMATE

Puzzles from the tropics

David Rind

THE reconstruction of changes in temperature and precipitation in equatorial Africa over the past 40,000 years, reported by Bonnefille *et al.*¹ on page 347 of this issue, addresses a problem of prime importance for our ability to predict future changes to the global climate.

Why is this so? In assessments of the anthropogenic influence on global climate in the coming century, the response of temperatures in the tropical regions represents perhaps the greatest uncertainty. The general circulation models (GCMs) used by different groups in estimating the degree of global warming to be expected from increased levels of atmosphere CO₂ give results for the tropics that differ by a factor of two (ref. 2). These differences affect the predicted change in latitudinal temperature gradients, and thus in storm tracks and intensities, in rainfall patterns — indeed, in the whole general circulation². More specifically, if the tropics warm significantly, there is the likelihood of a substantial increase in droughts³.

Although the reasons for the differences in the models' tropical response are not yet clear, they may be associated with the different convection or radiation schemes used. To decide which of the predicted responses is the most likely, researchers have turned to the palaeo-record — only to find that here the discrepancy is just as large. The 1981 CLIMAP⁴ data set indicated that, at the peak of the last ice age, tropical sea surface temperatures were relatively warm. This implies a low tropical sensitivity to severe climate perturbation. But the land-based glacial and pollen data indicate that the last ice age saw snowlines and vegetation zones lowered by about 1 km in mountains at a wide range of tropical and semi-tropical locations⁵. Assuming that this depression corresponds to a depression in temperature, and given a temperature drop with altitude of 5–6 °C km⁻¹, this would imply substantial tropical cooling. GCM results show that these land and sea temperature reconstructions are not consistent⁶, leaving considerable un-

reduced by one magnitude of extinction through the eclipse of a nearby hot star by a cloud (perhaps a cometary head), cooling of the shadowed region may cause its density to increase by two orders of magnitude in a fraction of a million years. This seems to provide a very promising mechanism for generating cometary tails and filaments □

A.P. Whitworth is in the Department of Physics, University of Wales, PO Box 913, Cardiff CF1 3TH, UK.

certainty about what the tropical climate was really like during the last ice age.

Looking further back in time, one finds that the record becomes even murkier. In the warm climate of the Mesozoic, the tropics seem to have been substantially warmer⁶, but during periods in the Cenozoic that were apparently very warm at high latitudes, there is little evidence of any warming in the tropics⁷. It is fair to say that until now the record has not helped us to understand how the tropics are likely to change in the future.

Bonnefille *et al.* address this issue by attempting to reconstruct the climate record for the Last Glacial Maximum at an intermediate altitude of about 2 km. This is an important difference from the glacial and pollen records that have been reported previously⁵, which have generally come from higher elevations. Furthermore, instead of simply using temperature lapse rates (variation of temperature with altitude) to generate a cooling estimate, they use modern analogues to relate the vegetational changes recorded in the pollen data to temperature and precipitation

Glacial Maximum, 4 ± 2 °C, is not sufficiently different from the lapse-rate estimate of 5–6 °C to be definitive, especially considering the uncertainties inherent in the authors' time-lagged approach. More importantly, as the authors note, they are considering a lower elevation than most other researchers. In the drier climate of the ice-age tropics, the atmospheric lapse rate would be expected to increase (to move somewhat closer to the dry adiabatic value of 10 °C km⁻¹ rather than the moist adiabatic value of 5 °C km⁻¹); this means that temperature changes should be somewhat smaller at lower elevations. A 4 °C cooling at 2 km is perfectly consistent with a 5 °C cooling at 4 km, and neither can be obtained at low latitudes from a GCM using the warm CLIMAP sea surface temperatures. So the results of Bonnefille *et al.* are actually quite consistent with greater tropical sensitivity. This is shown in the table, which presents the temperature changes as a function of altitude produced by the Goddard Institute for Space Studies GCM in the grid box that encompasses the tropical African observation point, for two sets of conditions: using the glacial sea surface temperatures deduced by CLIMAP and using those temperatures reduced by 2 °C. In both cases temperature depressions are larger at greater elevations; for the lower sea surface temperatures, the effect becomes larger because the atmosphere becomes drier and so the lapse rate increases.

This lapse-rate argument cannot be carried too far, however. For instance, one cannot assume that the tropics dried out sufficiently to increase the lapse rate until the warm sea surface temperatures become consistent with the substantial cooling aloft. The movement of glaciers down mountains is more difficult in a drier climate, and so requires a still greater temperature depression⁸. But lower tem-

TEMPERATURE CHANGE FOR THE LAST GLACIAL MAXIMUM IN THE GISS GCM FOR EQUATORIAL AFRICA

Altitude	ΔT (CLIMAP SSTs)	ΔT (CLIMAP – 2 °C)
Sea surface	–1.3°	–3.3°
2 km	–2.0°	–4.0°
4 km	–2.4°	–4.9°
6 km	–2.9°	–6.0°

Sea surface temperature changes are zonal averages for 4° S.

changes. This allows them to separate the effects of changes in temperature and precipitation on ecosystem distributions. They conclude that temperature changes derived from lapse-rate calculations using the pollen record overestimate tropical cooling by ignoring the fact that part of the pollen changes are induced by precipitation. Thus tropical cooling should be less than has been assumed, implying a lower tropical climate sensitivity.

Does this result settle the issue? Unfortunately not, although it is an important piece of evidence. Bonnefille *et al.*'s reduced cooling estimate for the Last Gla-

peratures would then mean an even greater lapse rate from high altitudes down to the warm surface, which would imply even drier conditions . . . and the whole argument regresses until there is no moisture left with which to form glaciers. For the same reason, it is unlikely that precipitation changes have caused most researchers to overestimate the pollen-derived cooling in other locations: the regions of glacial and vegetation-line descent are often contiguous, and the two phenomena have the same temperature requirement (cooling) but opposite moisture requirements. In principle, the use of both data

sets should allow the changes in temperature and moisture to be bounded.

How are we to solve this problem? We definitely need more studies like that of Bonnefille *et al.*, exploring tropical and semi-tropical palaeo-temperatures at a range of elevations and at widely separated locations. From the modelling end, we need to determine why the different GCMs produce different tropical responses to increases in CO₂. The uncertainty in tropical climate sensitivity emphasizes that we do not yet understand global climate sensitivity, and makes dynamical and regional predictions for the coming century that much more difficult. □

David Rind is at the Goddard Institute for Space Studies, NASA Goddard Space Flight Center, 2880 Broadway, New York, New York 10025, USA.

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Ann Bishop (1899–1990)

THE death on 7 May of Ann Bishop marks the end of an era during which the direction of British protozoology was influenced by three remarkable women — Doris Mackinnon (1887–1956), Muriel Robertson (1883–1973) and Ann Bishop herself.

A Manchester zoologist by training, Bishop worked all her life in Cambridge and made numerous and diverse contributions to protozoology including the nuclear and ciliary structural biology of the ciliate *Spirostomum* and various aspects of the biology of *Trichomonas* and *Entamoeba* species. She was involved in the discovery that the causative agent of blackhead in turkeys was *Histomonas meleagridis*, but it was as a malariologist that she became best known.

In the years overlapping the Second World War, Bishop made vitally important contributions to the understanding of drug resistance in malaria. In the late 1930s, as new antimalarial drugs were becoming available, there was great concern that these might easily evoke resistance as had happened with antibiotics. But there were few procedures or protocols that had been laid down for the investigation of this problem, and Bishop had to develop experimental systems to assess resistance. Her choice was *Plasmodium gallinaceum* in chickens, and by 1938 she had demonstrated resistance to proguanil. She also discovered the time at which resistance occurred, that it persisted even after

Trawling for receptors

Graham Warren

ENDOCYTOSIS is the process whereby substances on cell surfaces are enclosed in membrane-bound vesicles and taken into the cell. These substances then pass through the cell in what is usually thought of as an orderly itinerary of discrete stages (Fig. 1). But video images of living cells show that the endocytic pathway may be more interconnected than was previously thought. In the conventional picture, receptors that cluster in clathrin-coated pits in the plasma membrane pass through a series of internal, membrane-bound compartments¹, the first of which is the early endosome. Transferrin receptors are recycled almost immediately from this compartment back to the cell surface² whereas receptors for epidermal growth factor pass to late endosomes. In the process, these receptors become incorporated into budding vesicles that pinch off and congregate within what can then be called a multi-vesicular body³ (Fig. 2). These are processed further³ and are eventually delivered to lysosomes. There

is general agreement that each compartment in the endocytic pathway is a discrete, separate entity. In fact, it is difficult to see how parts of the pathway could function were this not the case. But on page 335 of this issue⁵, Hopkins *et al.* report observations that strongly suggest that the early part of the endocytic pathway is a network of interconnected tubules.

What Hopkins *et al.* have done is to use different fluorescent tags to follow both the transferrin and epidermal growth factor (EGF) receptors in living cells using video recording at low light levels. At steady state, the transferrin receptor reveals an interconnected, tubular network, through which occasional pulses of receptor appear to be moving at high speed (up to 1 $\mu\text{m s}^{-1}$). The network is very extensive but has not been seen before because it is labile: sensitive to low levels of ultraviolet light, lowered temperature and the chemical fixatives that are normally used to prepare samples for immunofluorescence and electron microscopy.

The effect of adding EGF is particularly dramatic as anyone who has seen the video will confirm. The network grows and the EGF receptors appear rapidly in boluses that move along the tubes at speeds of around 0.05 $\mu\text{m s}^{-1}$. Electron microscopy confirms that these boluses are multi-vesicular bodies (MVBs). The MVB has always been thought of as a discrete organelle and any tubular extensions have been short and thought to be

mosquito transmission and, more importantly, that there was cross-resistance between proguanil and other dihydrofolate reductase inhibitors which she later discovered extended to pyrimethamine. A decade later, with the availability of rodent malarias, she turned her attention to these and established procedures, which have now become standard in laboratories all over the world. Bishop also set herself the task of discovering how drug resistance occurs, a problem that remains unsolved. She also began to investigate the trigger for gametocytogenesis in malaria parasites, a question that 25 years later is still unanswered.

From 1942 to 1964, she was director of the Medical Research Council's Chemotherapy Research Unit at the Molteno Institute in Cambridge where many British and foreign scientists trained in experimental parasitology and benefited from her wide experience. Her work was recognized by her being elected to a Fellow of the Royal Society in 1959, a rare accomplishment for a woman at that time, especially for one engaged in essentially medical research. Those who knew Ann Bishop will remember her with affection as a great scientist and a gracious lady.

F. E. G. Cox

F. E. G. Cox is in the Immunology Section of the Division of Biomolecular Sciences, King's College London, Campden Hill Road, London W8 7AH, UK.

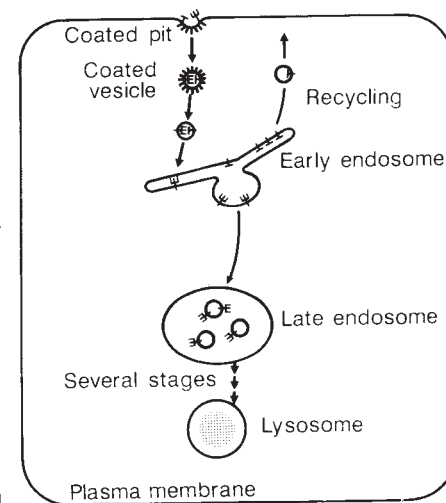


FIG. 1 The usual picture of receptor-mediated endocytosis. EGF receptors (—E) and transferrin receptors (T) are ingested by the cell and pass through a series of stages before being respectively destroyed or recycled. Multi-vesicular bodies can form at almost any stage from maturing endosomes or early lysosomes.

involved in removing the last of the recycling receptors^{6,7}. If it is not discrete but part of an extensive endocytic network through which it moves, then one's view of this organelle must change. One could instead think of it as a trawler that scours the tubular network for receptors, allowing transferrin receptors to pass through the net but trapping and internalizing a cargo of EGF receptors (Fig. 2). Movement of these MVBs through the network might be facilitated by a microtubule-based system⁸ and the net could be provided by whatever it is that makes up the coat responsible for budding into the MVBs.

Exciting though these observations are, they do pose many problems. The most obvious are those that are easily solved by discrete compartments but not so easily by networks. As an example, movement along the endocytic pathway is characterized by a fall in pH (ref. 9) which is easily understood if each compartment is dis-

crete and has different proton-pumping capabilities^{10,11}. In a network, one would need to place strong proton pumps in the membranes of the MVBs and sinks elsewhere, otherwise the pH would equilibrate rapidly throughout the network. It may even be that the network is not stable with time, but fragments and fuses in a manner similar to that of the endoplasmic reticulum¹². Those fragments containing the most proton pumps could then reach a lower pH than would be possible as part of a network.

It is also difficult to reconcile these observations with results obtained in other systems. To take just one example, using a polarized cell line, there is good evidence to show that the contents of early and late endosomes do not mix¹³, which either means that the cells have different endocytic pathways or that the terms 'early' and 'late' as applied to endosomes have different meanings in the two systems. This highlights one of the chief problems in the endocytic field — the absence of good markers for the early endocytic pathway. Until such markers are available one can only compare results

generated using different methods within one cell system. Meanwhile, the observations reported by Hopkins *et al.* provide a new view of the endocytic pathway and any future models will have to take these observations into account. □

Graham Warren is at the Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK.

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ACTIVE GALACTIC NUCLEI

A long, deep look at the shape of Seyfert galaxies

Julian H. Krolik

ACTIVE galactic nuclei are distinguished from normal galaxies principally by their tremendous power output from only a tiny volume of space. Another distinctive feature is the presence in their spectra of very strong and broad emission lines. The results, reported at a recent meeting*, of a worldwide campaign to monitor variations both in the emission-line and the ultraviolet continuum fluxes of a particular active galaxy, the Seyfert galaxy NGC5548 (refs 1–3), provide our first detailed picture of the density and physical state of the line-emitting gas in this type of object⁴.

The emission lines from active galactic nuclei are at once both mundane — in the sense that they are radiated by such common atoms and ions as H, C²⁺, C³⁺, N⁴⁺ and Mg⁺ — and dramatic, often carrying 10 per cent of the total power from the galactic nucleus and implying (from their Doppler shifts) gas velocities of up to 10⁴ km s⁻¹. By the mid 1970s, it was generally agreed that the lines derive their power via photo-ionization by the ultraviolet and X-ray continuum emission; and by the early 1980s there was also general agreement about the kind of physical conditions that exist in the line-emission region. Combining the latter inferences

with a measurement of the total ionizing power of the active nucleus, it is possible to estimate the distance between the continuum source and the line-emitting material. Direct experimental tests of this picture were lacking, however, as were data on the internal structure of line-emitting regions. The conference bore the happy news that both these problems have now been overcome.

The basic idea^{5,6} behind the work reported is that if the emission lines are powered by the continuum radiation, fluctuations in the continuum drive corresponding fluctuations in the local line emissivity within the emission-line region. The time required for a local readjustment of the ionization state and temperature following a change in the continuum is thought to be less than one hour, whereas the light travel time across the line-emission region is expected to be in the range from days to years, so fluctuations in the line flux should mimic delayed and smeared versions of the continuum fluctuations. If the fluctuations remain small enough to drive line emission linearly, the line light curve is related to the continuum light curve by a simple convolution with a response function. Deconvolution of the two light curves for the response function would then produce a one-dimensional map of 'marginal line emissivity' (the

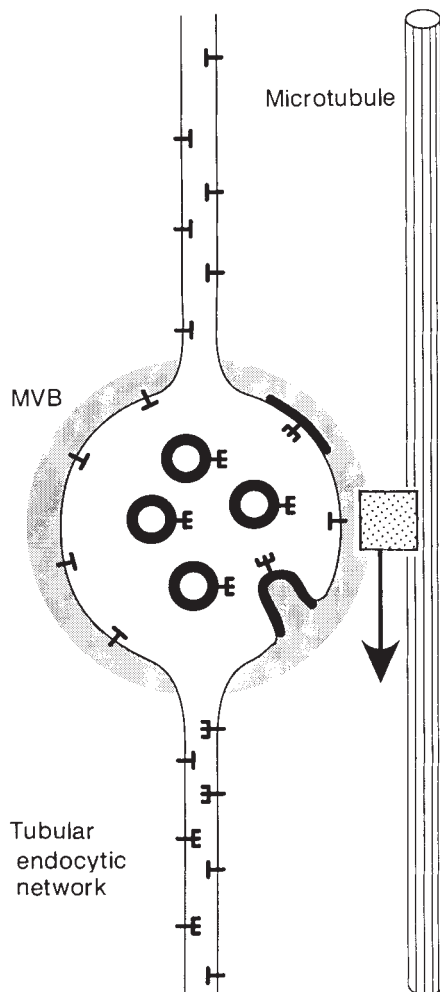


FIG. 2 Multi-vesicular bodies (MVBs) appear to be continuous with a tubular endocytic network and to move through these tubules using microtubule-dependent motors (arrow). As the MVBs move, one could imagine that EGF receptors (—E), but not transferrin receptors (T), would become incorporated into budding vesicles that pinch off and congregate within the lumen.

* Conference on Variability in Active Galactic Nuclei, Georgia State University, 2–4 May 1990.

partial derivative of the line flux with respect to the continuum flux, averaged over each paraboloidal surface of constant delay). Unfortunately this requires many observations to describe adequately the light curves, and the interval between observations must be chosen carefully to match the relevant timescales.

Such careful sampling is difficult to achieve in ground-based astronomy because observing is subject to uncontrollable interruptions, of which obscuration by the Moon's light and by bad weather are the worst examples. But orbiting satellites are subject to neither of these constraints, and so in 1987 a campaign was organized to use the International Ultraviolet Explorer satellite to observe the Seyfert galaxy NGC5548 every four days over a span of eight months. From this collection of spectra, time series for three continuum bands and eight emission lines were compiled. At the same time, a large-scale complementary ground-based campaign was mounted to gather data (albeit with less regular sampling) on the behaviour of the optical continuum and emission lines.

Ionization conditions

With these data in hand it has become feasible to attempt the deconvolution. The result is that (at least in this example) the lines fall neatly into two groups: those radiated in relatively high-ionization conditions (H II, N V, C IV, Si IV and Lyman α) and those from lower-ionization conditions (C III, Mg II, O I and H β). The first set responds most strongly to changes in the continuum at zero lag, the response tapering off almost to zero after 20 days. The second set responds after a considerably greater delay, but only for the C III and H β (ref. 7) lines was the signal-to-noise ratio high enough to permit a good look at the response function: for both of these lines, the response at zero delay is quite weak, builds to a peak after 20 to 30 days, and falls steeply at larger lags.

Such one-dimensional maps are interesting, but they are less than satisfactory because they give the marginal emissivity only as a function of delay along our line of sight. Whatever symmetry truly characterizes the emission-line region, it is very unlikely to be defined by our line of sight. The shapes of the response functions, however, give a good guide to the true symmetry. When the lines are radiated isotropically (a good approximation for many of those measured), response functions peaking at zero lag must be associated with quasi-spherical regions, whereas the simplest symmetry for those peaking at larger lags is cylindrical. Thus it seems that (at least in NGC5548) the emission-line region has two sub-regions: an inner, roughly spherical zone responsible for the high-ionization lines and an

outer, flattened zone responsible for the low-ionization lines.

The fact that five response functions are obtained from the same (high-ionization) region permits a still more detailed analysis. At each lag τ , the overall amplitude of the high-ionization response functions reveals the solid angle subtended by the highly ionized material on a sphere of diameter $c\tau$; the ratios of these functions reveal (with the help of some atomic-physics calculations) the physical conditions in the gas. Adding into the analysis the mean ionizing power, a new estimate may be made of the distance from the continuum to the line-emitting region; satisfyingly, this distance corresponds to the radius of the sphere ($c\tau/2$) to within a factor of 2–4. Such self-consistency adds considerable confidence to the inferences drawn about physical conditions: the high-ionization line-emitting material in NGC5548 is spread over spherical shells ranging in radius from 4 to 14 light-days, with the greatest amount of line radiation coming from the shell with a radius of about 8 light-days; the pressure stays fairly constant throughout this region, maintaining a density of around 10^{11} H atoms cm^{-3} ; and the thickness of individual line-emitting clumps is less than about 10^{11} cm.

Because only two lines are available to constrain the models, the structure of the outer (low-ionization) region is determined less well. Although it must be flattened cylindrically, the response functions could correspond equally well to a ring with a radius of about 25 light-days lying face-on to our line of sight, or a ring with a radius of about 100 light-days viewed almost edge-on.

These studies are producing a higher level of understanding of active-galaxy emission lines. Both direct confirmation of inferences from models and detailed structural information are now accessible, provided that the necessary large-scale coordinated efforts can be mounted. Judging by the number of new monitoring programmes being promoted at the meeting, that is a cost that will soon be met. $\square$

Julian H. Krolik is in the Department of Physics and Astronomy, Johns Hopkins University, Baltimore, Maryland 21218, USA.

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Equilibrated junk

WHAT should we do with our rubbish? Dumping it as landfill is increasingly unacceptable, clear-air lobbyists oppose incineration even at sea, and few citizens are dedicated enough to sort and classify the stuff for recycling. Daedalus recalls composting, that ancient method of reducing organic waste to a uniform and fertilizing humus. He is now adapting the technique to urban rubbish.

His idea is simply to heat the rubbish to such a temperature that all possible chemical reactions between its components go to completion. The end product, DREADCO's 'Rumus', would be the stablest, lowest-energy compound or mixture of compounds that could be formed from the initial composition. The concept will be familiar to those astrochemists who calculate what minerals would preferentially separate from any assumed primaeval planetary material as it cooled. Rubbish presents a harder challenge. The only safe bets are that Rumus will be extensively chemically bonded, and will therefore be a solid; and since it has the lowest energy possible for its composition, its formation will give out heat.

So while DREADCO's chemists wrestle with thermodynamic equilibria in rubbish, its chemical engineers are making Rumus in practice. They are compacting carefully defined mixtures of plastic bags and bottles, old newspapers, tin cans, broken china, disposable nappies and takeaway-meal residues at high temperatures and pressures, measuring the heat evolved and studying the product. From the results, they hope to design a pilot plant to carry out this self-heating reaction automatically and continuously.

Raw Rumus will be an uninspiring, brownish solid. At first Daedalus hoped that it might be hard and strong enough for road-stone or breeze blocks. But he then recalled that a mixture always has a lower melting point than its ingredients. Rumus will be such a horrendous mixture that its melting point will be quite low. Inert, softish and yielding, it will be ideal for asphalt, roof sealants, caulking and the like. It may, of course, contain two or more intermixed phases of different melting point, thus being self-reinforced into a tough, useful solid.

But to command a wide and varied market, it will probably need a bit of modification. The incoming rubbish may have to be spiked with conditioners like sulphur, to vulcanize and toughen the organic components; or silicon, which should combine with metals, organics and inorganics, to merge otherwise immiscible phases. Once the process has been optimized, DREADCO's Rumus will solve the whole refuse disposal problem, and make a splendid profit into the bargain. David Jones

On the edge of a wing

SIR—It is very striking how the wings of all species of the fruitfly *Drosophila* have a similar, elliptical shape, independent of their size. This suggests that there could be a standard growth mechanism with parameters that vary within narrow limits.

D'Arcy Thompson¹ showed that the shapes of organs of various plants and animals can be accurately described using geometric figures defined by simple equations. To characterize the variation in size and shape of *D. mediopunctata*'s wing, we tried to adjust an ellipse to its contour. When we overlayed the adjusted ellipse on a photograph of the wing, we were impressed by the nearly perfect fit (see figure).

Because the shape and size of an ellipse can be precisely described by two independent parameters (in our figure *SH* and *SI*), we can characterize the shape and size of a wing using the parameters of the adjusted ellipse. We did this when we

analysed the wings of 17 females collected from the field and of 42 of their daughters raised in the laboratory, half at 16.5 °C and the other half at 20 °C. Comparing the three groups, we found no significant difference in the shape of their wings ($F=0.91$; $P>0.9$; d.f.=2, 56) although there were clear and significant differences in size ($F=18.81$; $P<0.001$; d.f.=2, 56); showing that, despite the great variation in size, there is little variation in the wing shape.

We also adjusted ellipses to the wings of a few animals of other species: *D. atrata*, *D. quadrum*, *D. hydei*, *D. mulleri*, *D. willistoni* and *D. melanogaster*. Similarly, we examined published photographs of the wings of 17 *Zygothrica*² species and of 16 species and 40 hybrids of the picture-winged Hawaiian *Drosophila*³. In all cases, there was a good fit for the adjusted ellipses.

Together with use of genetic information available for *Drosophila*, the use of ellipses opens the possibility of new experimental investigations on questions related to size and shape. These could include evolution in species of *Drosophila* and related genera, environmental and

genetic factors and the effects of selection for size on shape. We suspect that the wings of other insects may also be described by simple geometric figures, although not necessarily ellipses.

LOUIS B. KLACZKO

BLANCHE C. BITNER-MATHE

Depto. Genetica,
Inst. Biologia Universidade Federal do
Rio de Janeiro,
21949 Rio de Janeiro, Brazil

Channel hands

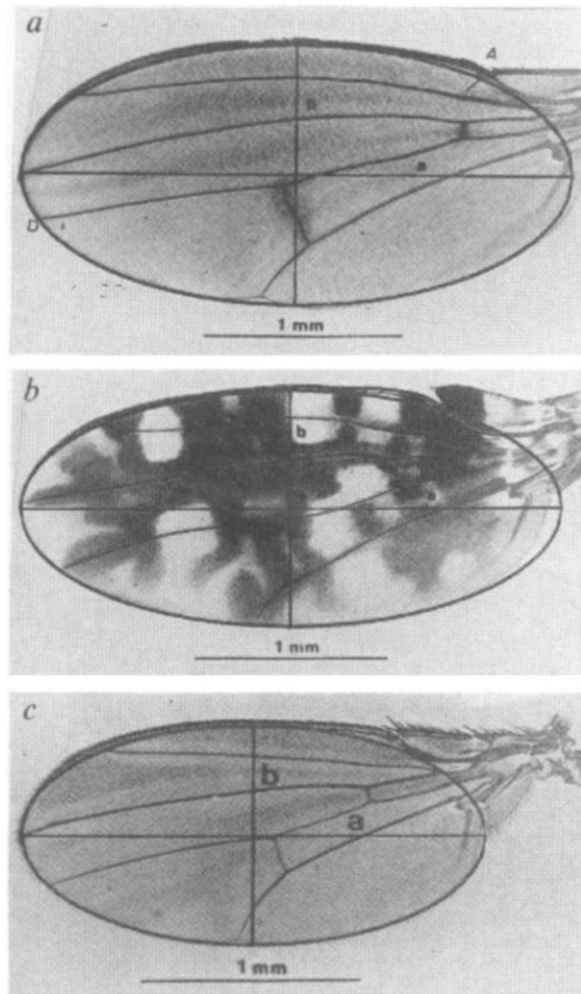
SIR—Calcium-binding proteins are grouped into several classes, one of which consists of intracellular transducers of calcium signals. These calcium-modulated proteins contain sequences of 29 amino acids, the EF hand which forms an α -helix, a calcium-binding loop and a second α -helix¹.

The deduced amino-acid sequences of many voltage-gated ion-channel proteins have been reported recently. Sodium-channel α_1 and dihydropyridine-sensitive calcium channel α_1 polypeptides have recognizably similar amino-acid sequences, both of which probably evolved by two gene duplications from a polypeptide resembling the *Drosophila Shaker* gene product, a voltage-gated potassium channel^{2,3}. In the course of work on brain calcium-binding proteins, I found that all three calcium-channel α_1 -subunit and seven of eight published sodium-channel α_1 -subunit sequences contain one or more putative EF hands. The exception is the *Drosophila* DSC locus⁴, but another probable *Drosophila* sodium-channel α_1 -subunit which has been partially sequenced may have the conserved EF hand⁵. By contrast, none of the 10 potassium-channel sequences so far published contains apparent calcium-binding sites.

All the sodium-channel α_1 -subunit sequences except the *Electrophorus* sequence contain a conserved region which scores 10 or 11 in the Tufty-Kretsinger test and all scores can be improved to 12 or 13 by allowing plausible test modifications described for other EF hands. Other criteria for calcium binding⁶, such as the presence of three acidic ligands and the absence of more than one serine or threonine serving as a ligand in the calcium-binding loop, are fulfilled by these sequences. Critical positions are better conserved than are noncritical ones. Polypeptides from *Electrophorus* electric organ and rat skeletal muscle also contain a putative EF hand downstream.

The calcium channel α_1 -subunit sequences score higher in the EF-hand test than do the sodium-channel sequences and they have more characteristics of EF hands, particularly the specified I, G and first E. The sequences of the rat aorta and brain calcium-channel α_1

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a, Wing and adjusted ellipse of *Drosophila mediopunctata* ($a=1.398$ mm, $b=0.653$ mm, $SI=\sqrt{ab}=0.955$ mm, $SH=0.4668$, $r=0.9998$). b, Wing of *D. quadrum* ($a=1.4156$ mm, $b=0.612$ mm, $SI=0.931$ mm, $SH=0.4324$, $r=0.99933$). c, Wing of *D. melanogaster* ($a=1.054$ mm, $b=0.496$ mm, $SI=0.723$ mm, $SH=0.4707$, $r=0.9996$). We took at least 20 points along the outline of the wing (using the internal edge of the marginal vein from A to D). We fit the general equation of the ellipse to the cartesian coordinates of these points using a least-squares procedure. Solving this equation for the x-observed values, we calculated a correlation (r) between the observed and expected y-values, and the centre and orientation of the ellipse found. Through a translation and rotation of the coordinates system, the general equation was transformed to $x^2/a^2 + y^2/b^2 = 1$, where a and b are the radii of the two director circles of the ellipse. Their geometric mean is the size parameter ($SI=\sqrt{ab}$); the shape parameter is the proportion of the smaller to the larger radius ($SH=b/a$).

Amino-acid sequences in sodium- and calcium-channel α_1 subunits examined by the Tuftý-Kretsinger EF-hand test. Ligand vertices for calcium coordination (X, Y, Z, -Y, -X, -Z) as described in ref. 1 are indicated. In the test sequence, E, G and I are specified where indicated: n, a hydrophobic residue (L, I, V, F, M); asterisk, an oxygen-containing amino acid (D, N, E, Q, S, T). Amino acids satisfying the test are underlined. Plausible modifications to the test are double-underlined with the increased scores in parentheses. Origins of these sequences are as follows: Eel, *Electrophorus electricus* electric organ⁹; Rat I and rat II, rat brain¹⁰; Rat III, rat brain¹¹; Rat IIA, rat brain¹²; Rat S, rat skeletal muscle¹⁴; Rat C, rat cardiac muscle¹⁵; Rab S1, rabbit skeletal muscle¹⁶; Rab C, rabbit cardiac muscle^{17,18}; Rat A, rat smooth muscle (W. J. Koch, unpublished); Rat B, rat brain (A. Hui, unpublished).

Vertices:		X	Y	Z	-Y	-X	-Z		Scores				
T-K TEST:	E	n	n	n	*	G	I	E	n	n	n	(of 16)	
Na ⁺ channel α_1 subunit upstream sequences:													
Eel	1588	<u>M</u>	<u>E</u>	<u>I</u>	<u>W</u>	<u>H</u>	<u>K</u>	<u>E</u>	<u>D</u>	<u>P</u>	<u>I</u>	1616	9(12)
Rat I	1607	<u>M</u>	<u>E</u>	<u>I</u>	<u>W</u>	<u>H</u>	<u>K</u>	<u>E</u>	<u>D</u>	<u>P</u>	<u>I</u>	1635	10(13)
Rat II	1797	<u>M</u>	<u>E</u>	<u>I</u>	<u>W</u>	<u>H</u>	<u>K</u>	<u>E</u>	<u>D</u>	<u>P</u>	<u>I</u>	1825	11(13)
Rat III	1743	<u>M</u>	<u>E</u>	<u>I</u>	<u>W</u>	<u>H</u>	<u>K</u>	<u>E</u>	<u>D</u>	<u>P</u>	<u>I</u>	1771	11(13)
Rat IIA	1797	<u>M</u>	<u>E</u>	<u>I</u>	<u>W</u>	<u>H</u>	<u>K</u>	<u>E</u>	<u>D</u>	<u>P</u>	<u>I</u>	1825	11(13)
Rat S	1612	<u>M</u>	<u>E</u>	<u>I</u>	<u>W</u>	<u>H</u>	<u>K</u>	<u>E</u>	<u>D</u>	<u>P</u>	<u>I</u>	1640	11(12)
Rat C	1795	<u>M</u>	<u>E</u>	<u>I</u>	<u>W</u>	<u>H</u>	<u>K</u>	<u>E</u>	<u>D</u>	<u>P</u>	<u>I</u>	1833	11(13)
Na ⁺ channel α_1 subunit downstream sequences:													
Eel	1642	<u>K</u>	<u>I</u>	<u>S</u>	<u>Y</u>	<u>L</u>	<u>D</u>	<u>V</u>	<u>L</u>	<u>L</u>	<u>A</u>	1670	10(11)
Rat S	1668	<u>K</u>	<u>I</u>	<u>S</u>	<u>Y</u>	<u>L</u>	<u>D</u>	<u>V</u>	<u>L</u>	<u>L</u>	<u>A</u>	1694	10(11)
Ca ²⁺ channel α_1 subunit sequences:													
Rab S1	1401	<u>E</u>	<u>E</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>W</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>P</u>	1429	11(14)
Rab S2	1401	<u>E</u>	<u>E</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>W</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>P</u>	1429	11(14)
Rab C	1401	<u>E</u>	<u>E</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>W</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>P</u>	1429	11(14)
Rat A		<u>E</u>	<u>E</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>W</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>P</u>	1429	11(14)
Rat B		<u>E</u>	<u>E</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>W</u>	<u>A</u>	<u>E</u>	<u>D</u>	<u>P</u>	1429	11(14)

subunits in this region are very similar to the rabbit skeletal muscle sequences (W. J. Koch and A. Hui, unpublished data). The putative EF hand linked to the IVS6 region 25 amino acids upstream is one of the most highly conserved areas in the entire polypeptide with only two noncritical (for EF hand formation) changes. No downstream EF hands were detected in these sequences. Overall, only 2 of 29 amino acids are identical across all 12 sequences, so it is surprising that the structural features (supported by secondary-structure predictions; ref. 6 and unpublished data) of a particular kind of calcium-binding site should be retained.

The high degree of conservation in these sequences suggests that the putative EF hand(s) are important in channel structure and/or function. This region is internal and probably close to the lip of the channel. Because these sequences occur in sodium and calcium channels and not potassium channels, calcium binding could be involved in some aspect of depolarization and not repolarization or facilitation of ion permeation. Perhaps calcium binding is part of an inhibitory mechanism to close or inactivate these channels consequently limiting depolarization and cation influx. If, on the other hand, the sodium-channel sequences do not bind calcium

ions with micromolar affinity, then the calcium-channel sequences could be the high-affinity internal calcium binding site which facilitates ion permeation^{7,8}.

JOSEPH BABITCH

Chemistry Department,
Texas Christian University,
2900 S University Drive, Fort Worth,
Texas 76129, USA

Gas discharge at Lake Nyos

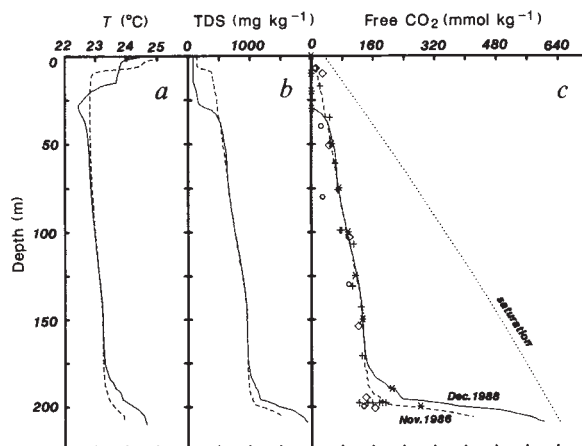
SIR—An outburst of lethal gas from Lake Nyos killed about 1,700 people on 21 August 1986¹. It has been confirmed^{1,2} that the gas was almost pure CO₂ of magmatic origin, but the volume of gas released, the mechanism of the sudden release and the likelihood of a future recurrence remain open questions. We conducted a survey in December 1988 to measure temperature, conductivity and CO₂ profiles in the lake, which has enabled us to estimate the influxes of heat and dissolved materials, and to estimate a recurrence time for such a gas discharge.

We measured temperature and conductivity profiles with a CTD (conductivity-temperature-depth) profiles; gas concentrations were measured by collecting water samples from various depths using a 1.4-l Niskin bottle with a plastic gas bag attached². Samples below 50 m contain constant relative concentrations of Mg²⁺, Fe²⁺, Ca²⁺, Na⁺, NH₄⁺, K⁺, HCO₃⁻ and Si. These components were summed for each sample to convert measured conductivity to concentration of total dissolved solids (TDS). Temperature and TDS profiles in December 1988 are compared with

those obtained in November 1986³ (a and b respectively in figure). The increase in both temperature and TDS below 165 m is significant, and supports the view that the lake is fed by an inflow of warm, CO₂-charged, mineralized water^{2,4,5}. Integrating the temperature and TDS profiles below 165 m gives heat and TDS fluxes of 0.36 MW and 1,064 tonne yr⁻¹, respectively.

Complete sampling of both gas and water is difficult because the deep water contains a very high concentration of free CO₂ even after the 1986 gas release. We have developed a simple syringe sampler with which CO₂ can be fixed *in situ* in a solution of NaOH. Sampling for five depths between 50 m and 150 m gave total CO₂ concentrations that increase linearly with TDS. Free CO₂ concentrations, given by the difference between total CO₂ and HCO₃⁻ concentrations, are plotted in c of the figure together with data obtained between October 1986 and May 1987^{2,4}. Our free CO₂ data for the mid-depth water (50–165 m) were smoothed using the linear CO₂-TDS relationship. Over the two years, there has been no change in free CO₂ concentration at mid-depths.

For depths greater than 175 m, the high CO₂ concentrations made proper sampling difficult. Fortunately, however, we were able to recover 7.08 l of gas from a depth of 190 m comprising over 99.8% CO₂ (refs 5,6) which completely exsolved into the collection bag, indicating a free CO₂ concentration of 209 mM. In the sample from 200 m, the exsolved gas exceeded the capacity of the 10-l bag, implying that the free CO₂ concentration was higher than 283 mM. The free CO₂ concentrations at 190 and 200 m indicate that the CO₂/TDS ratio is higher below



a, Temperature profiles in Lake Nyos. Solid line: our data for 16 December 1988. Dashed line: data for 8 November 1986³. b, TDS profiles in Lake Nyos calculated from the correlation between CTD conductivity (temperature-corrected) and TDS in the sampled water. c, Measured and estimated free CO₂ concentration in Lake Nyos. Measured concentrations indicated by circles (October 1988⁴), squares (November 1986³), crosses (January, March and May 1987⁴) and stars (December 1988, our data). The 200-m data point for December 1988 indicates an approximate minimum free CO₂ concentration (see text). Solid (our data) and dashed (ref. 3) lines smoothed free CO₂ profiles based on the TDS profile and CO₂-TDS relationship. Dotted line, saturated concentration⁵ of free CO₂ for the pressure and temperature conditions of the lake.

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165 m than at mid-depths. We assume that much of the total dissolved CO_2 was released in the 1986 gas outburst and that non-gaseous components were retained; thus the deep-water CO_2/TDS ratio is higher because it is being replenished from a bottom spring rich in CO_2 .

If CO_2 concentrations in the deep water result from mixing of mid-depth water and bottom spring water, the CO_2 -TDS relationship for the deep water can be assumed linear, with a slope that can be estimated from data points at 150 m and 190 m. We used this slope to convert the TDS profile to the CO_2 concentration profile in the deep water (*c* in figure). We analysed Tietze's CTD data³ in a similar fashion. The smoothed free CO_2 profile (see *c* in figure) suggests that the bottom water is approaching CO_2 saturation. We calculate an average free CO_2 flux over the past 25 months of $515 \times 10^6 \text{ mol yr}^{-1}$ ($0.012 \text{ km}^3 \text{ STP CO}_2 \text{ yr}^{-1}$). Based on this CO_2 flux and a revised bathymetry⁷, the hypolimnion (depths below 30 m) of Lake Nyos may become saturated with free CO_2 within about 50 years if the estimated CO_2 flux remains unchanged. Frequent measurements of water and gas compositions of the lake would contribute to more accurate evaluation of CO_2 fluxes, essential to assess the possibility of a future gas release.

YUKIHIRO NOJIRI*

National Institute for Environmental Studies,

Tsukuba, Ibaraki 305, Japan

MINORU KUSAKABE

Okayama University,
Misasa, Tottori 682-02, Japan

JUN-ICHI HIRABAYASHI

Tokyo Institute of Technology,
Kusatsu, Gumma 377-17, Japan

HIROAKI SATO

Hiroshima University,
Hiroshima 730, Japan

YUJI SANO

University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan

HIROSHI SHINOHARA

Tokyo Institute of Technology,
Meguro-ku, Tokyo 152, Japan

THOMAS NJINE

University of Yaounde,
Yaounde, Cameroon

GREG TANYILEKE

Institute for Geological and Mining
Research,
Yaounde, Cameroon

*Full addresses available from Y.N.

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Natural habitat of coelacanths

SIR—Fifty years after the discovery of living coelacanths, these ancient fish are threatened with extinction within two decades¹. Can this famous fish be protected and saved?

Almost all (98.6 per cent) of coelacanths have been caught along the west coast of Grande Comore, Western Indian Ocean (the site where most of these fish have been captured)². Hardly any have been found on the east coast. Last year, we carried out a submersible operation to

indeed a very small number.

Among the encountered individuals, only two were 60-80 cm long, and 5 per cent are smaller than 100 cm. Size at birth is probably 40 cm. The two juveniles were observed together with one large female, suggesting that their habitat is similar to that of adults. Official catch records reveal that 3 of 43 individuals (7 per cent) were smaller than 100 cm. Low numbers of juveniles can result from low birth rate and/or high juvenile mortality. Coela-



Coelacanths, shy but present.

study the extent of the population and surveyed 9 per cent of the area thought to be the coelacanths' habitat (see map).

During daytime, coelacanths are cave dwellers and aggregate in small peaceful groups, the number of members changing daily. Each coelacanth occupies a home range of at least 8 km of coastline. At this site, there are several occupied caves between 180 and 210 m in depth. At night, coelacanths leave their caves and hunt singly for fish. The number of caves seems to limit their distribution. Below 220 m depth, caves are rare and prey fish are less abundant. On the other hand, temperature dependent oxygen saturation of the blood³ probably prevents the occupation of shallower warmer waters.

We found 38 individuals. We recorded 19 as residents since they were repeatedly encountered. Assuming that this sample is representative, the total population of the west coast can be estimated as 210 individuals. By an alternative method, repeated observation of 6 occupied caves revealed an average of 13.5 coelacanths or 150 individuals, for the entire coast. These are first estimates for the coelacanth population at Grande Comore. The small differences between both estimates suggests that these are reliable numbers. If the highest number of observed coelacanths per cave is taken as an indication for cave saturation, 370-520 individuals would be the habitat's carrying capacity at Grande Comore, which is

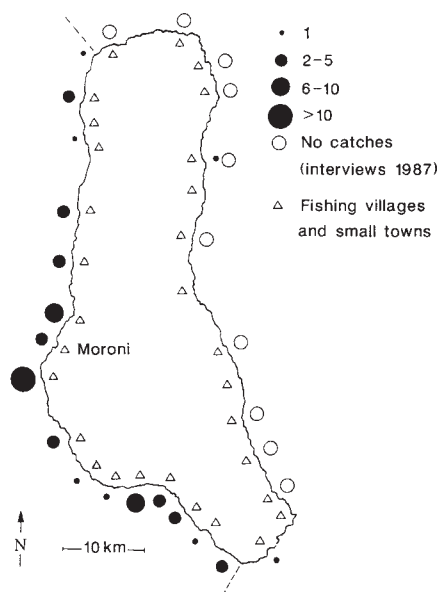
canths are ovoviviparous, fecundity probably being low and the gestation period more than 12 months. Nothing is known about longevity, growth or age at maturity, or size, age structure and sex ratios of populations. Although our present knowledge of life-history parameters is incomplete, coelacanths seem to be highly K-selected organisms with a low production of offspring and an apparently high longevity. Therefore, the small population size we observed should be a cause for concern.

Official catch records for the past 28 years from Grande Comore show that 2.6 ± 1.5

(s.d.) individuals (or 1-1.5 per cent of the total population) are annually eliminated by human fishing. Over the years, the catch rate of coelacanths has remained stable although local fisheries have expanded. This could indicate that the coelacanth population is already in decline. But increased fishing efforts do not necessarily imply that there has been proportional increase in fishing for coelacanths. Coelacanths are caught at night from small outrigger canoes as an incidental bycatch, and we have found no evidence that night fishing has increased. Furthermore, the rapid developments of the pelagic fishery outside the coelacanths' habitat do not harm the coelacanth population. If the estimations of the carrying capacity are correct, then the low population size could have existed for centuries. For many years coelacanths have been caught in very small numbers, and therefore the shift from traditional to modern fishery has not affected the population.

In the future, we should carefully monitor the number of coelacanths in this region. Details of life-history parameters need to be studied to predict changes in the population. Coelacanths are rare, and concentrated international efforts should be made to protect them. Threats to their survival might arise from commercial interests such as public aquariums.

Since September 1989, trade of living coelacanths is strictly forbidden by a decree



Map of Grande Comore (capital Moroni) with locations of coelacanth catches (filled circles) and distribution of coastal fishing villages and small towns (open triangles). The west coast between Mitsamiouli and Chamboini (dotted lines) is the coelacanth habitat.

of the late president of the Comores. In October 1989, coelacanths were upgraded to schedule 1 of the conservation charter CITES. This might ensure that future generations can rejoice in these gentle and peaceful survivors of our own vertebrate history.

HANS FRICKE
KAREN HISSMANN

Max Planck Institut für
Verhaltensphysiologie,
D8130 Seewiesen, FRG

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Too soon for consensus?

SIR—Crumpton and Dedman in their *Scientific Correspondence* (*Nature* **345**, 212; 1990) proposed a common nomenclature system for a group of proteins presently referred to as lipocortins, annexins, calpactins and so on. Several of us, including those who first identified, isolated and cloned several members of this family, do not agree fully with these changes.

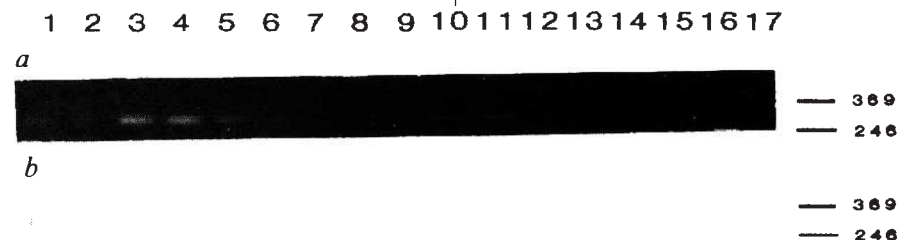
We have no objection to the use of the general designation of annexin to describe the group. But because there is no clear, universally accepted function for any of the proteins we believe that a new nomenclature is premature. Because each family member probably has a specifically tailored function, we suggest, at least in the interim, that a common universally accepted name for each protein (calpactin, endonexin and so on) would simplify matters.

One member of this family has been termed lipocortin-1 in most papers pub-

Improving PCR efficiency

SIR—The polymerase chain reaction (PCR) is a very sensitive *in vitro* DNA amplification method¹. Therefore, carry-over of even minute quantities of reaction products can lead to serious contamination problems and false-positive results. Tidiness and adherence to a strict set of protocols can avoid disaster^{2,3}. Methods

used the amplification of a 275-base pair fragment of the *elt* gene of enterotoxigenic *Escherichia coli* ATCC 37218 (ref. 5). We added 5–50,000 lysed bacterial cells to individual reaction mixtures (excluding *Taq* DNA polymerase), followed by 0.5 units of DNase I or 10 units of *MspI*. After incubation and subsequent inactivation of



Decontamination efficiency of DNase I or *MspI* treatment in PCR. (See ref. 5 for standard methods.) Reaction mixtures (excluding *Taq* DNA polymerase) which contained lysed bacteria as artificial contaminations were treated with 0.5 U DNase I for 30 min at room temperature or with 10 U *MspI* for 1 h. After incubation, enzymes were inactivated by boiling, *Taq* DNA polymerase added and 35 cycles of PCR performed. a, ethidium bromide-stained 1.5% agarose gel; b, Southern blot of 10 µl PCR product. Lanes: 1, 50 *E. coli* ATCC 37218, no DNase I or *MspI* treatment of reaction mixture; 2, reaction mixture *MspI* treated before adding 50 bacteria; 3–9, contamination of reaction mixture with 50,000, 5,000, 500, 50, 10, 5 and 0 bacteria, respectively, *MspI* treatment; 10, reaction mixture DNase I treated before adding 50 bacteria; 11–17 contamination of mixture with 50,000, 5,000, 500, 50, 10, 5 and 0 bacteria, respectively, DNase I treatment.

which eliminate contamination by PCR products from previous amplifications are indispensable, especially for diagnostic applications. As recently reported in *Scientific Correspondence*⁴, such contaminations can be eliminated from reaction mixtures by ultraviolet treatment before addition of target DNA.

We have developed an alternative method in which treatment of individual reaction mixtures before adding template DNA and *Taq* DNA polymerase with DNase I or restriction endonucleases that cut internal to the pair of amplification primers prevent amplification of contaminating DNA. As a model system, we

enzymes, we added *Taq* DNA polymerase and performed 35 cycles of PCR. As shown in the figure, *MspI* treatment reduces contamination by a factor of 5 to 10 without impairing the efficiency of the PCR. DNase I treatment reduces contamination by a factor of 1,000. Comparison of lanes 1 and 10 shows that the efficiency of PCR is not reduced by this treatment as primers are not significantly attacked by DNase I under the conditions chosen. The decontamination conditions can easily be optimized together with optimization of PCR components.

The efficiency of restriction-enzyme digestion can be increased by adding more than one enzyme and by increasing the enzyme concentration as well as the incubation time. The sequence specificity of the restriction-enzyme treatment can be circumvented by using a cocktail of enzymes that recognize tetranucleotide target sequences. The main advantage of this method is its general applicability based on the use of DNase I, a cheap enzyme requiring no sequence information between amplification primers.

B. FURRER
U. CANDRIAN
P. WIELAND
J. LÜTHY

Laboratory of Food Chemistry,
Institute of Biochemistry,
University of Berne, 3012 Berne,
Switzerland

J. BROWNING
B. PEPINSKY
B. WALLNER

Biogen Inc.,
14 Cambridge Center,
Cambridge, Massachusetts 02142, USA

R. J. FLOWER
S. H. PEERS

Dept of Biochemical Pharmacology,
St Bartholomew's Medical College,
London EC1M 6BQ, UK

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A definite maybe

E. I. Hamilton

Deadly Deceit — Low Level Radiation, High Level Cover-Up. By J M Gould and B A Goldman. *Four Walls Eight Windows*, New York: 1990. Pp. 213. \$19.95.

How Safe is Safe? Radiation in our Daily Lives. By Barrie Lambert. *Unwin*: 1990. Pp. 275. Pbk £7.99.

Two popular concepts in radiological protection are that all ionizing radiation is harmful to living cells and that there is a linear relationship between dose and effects. But some recent studies indicate that these concepts may not apply to people exposed to low doses of radiation. The significance of radiation risks is largely a polemical issue — for low doses (1–10 millisieverts per year) the evidence for presumed effects is largely of a statistical nature and is not always supported by epidemiology.

Gould and Goldman present a statistical evaluation for excess deaths in the United States which they relate to exposure from low-level radiation (the levels are not given) following releases of radioactivity into the environment from global fallout, nuclear accidents and from nuclear establishments. They consider reported excess deaths in the United States to be high — for example, 40,000 after the arrival of Chernobyl fallout. Those most susceptible are the very young, the very old and those suffering from infectious diseases. A comprehensive demographic breakdown of mortality and morbidity statistics is not provided by Gould and Goldman, neither do they mention the radiation dose received by individuals, nor the radiobiological effectiveness of the radiation. Instead they simply relate excess deaths with concentrations of radionuclides in air, water and food. The apparent delay between insult and effect (death) is usually only a few months; such a feature is unknown in radiological protection except following exposure to extremely high doses of radiation. The cause underlying the effects of exposure is considered to be an impairment to the immune system caused by free radicals formed in tissues following the passage of radiation. The authors comment on the presumed high levels of fallout in central Africa in the 1950s and the emergence of AIDS (or should this be HIV?), together with the recent increase in other immune-response diseases such as *Candida albicans* infections, Lyme disease, chronic Epstein–Barr virus infections, septicaemia and an impairment of mental and scholastic achievement, all of which the authors say have increased in the United States since nuclear-weapons testing first started.

The authors use naive statistics and an inadequate database, paying insufficient

attention to the role of demographic variables common to the subset of exposed individuals in relation to the general population. They do not consider

which are known to constitute a risk to health (somatic and genetic), singling out low levels of radiation as the most important cause of excess death and disease is not justified. Perhaps today, through poorly understood interactions and the pervasive distribution of chemicals (both man-made and natural) in the environment has produced a matrix in which transferable mutagenic changes can occur, and this could be one of the costs of technological advance for which technical remedies will eventually need to be forthcoming.

The future of the nuclear industry, and

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Food for thought — picnicking at Sellafield nuclear reprocessing plant.

modern statistical approaches to complex issues such as hazard models developed by Cox. They do not discuss possible effects of low-level doses from natural sources, or localized enhanced levels such as those associated with the mining of uranium ores, for example at Katanga, central Africa or within the Wismuth–Jochamstal region of central Europe. Neither have they considered the morbidity and mortality data following exposure to radon in houses. All these sources are relevant to any consideration of low-dose effects from ionizing radiation. The authors have extrapolated results of recent animal experiments far beyond that which is reasonable, even though some studies do indicate an involvement between radiation and the immune system. They also cite examples where federal agencies are accused of suppressing or manipulating data, a practice which is not unique to radiation issues.

Considering all the industrial products now present in the environment, some of

especially the production of electricity, requires a deeper understanding both by the general public and governments. The many controversial issues need to be discussed openly and both the risk and advantages set in perspective. Lambert provides an excellent account which is intended to allay fears and to explain some of the controversial issues, based upon scientific evidence rather than scientifically baseless claims. All the main issues, ranging from measurement of radiation, its effects, estimation of risks, nuclear reprocessing and discharges of radionuclides into the environment, are discussed. The pros and cons of the various arguments are stated in a fairly impartial way; those areas where uncertainties still exist and when policies have been suspect are highlighted.

Radiobiological aspects are treated in a comprehensive manner but the discussion of environmental distributions of radionuclides is weak, which is a pity as many pressure groups focus their concern upon

the presence of man-produced radio-nuclides in the environment. For the general public it is important to get the message across that today there is a sound basis for proliferation of nuclear power; there are no insurmountable scientific problems in the disposal of waste products to the sea or land. There are risks associated with all technologies, but there are also considerable advantages — hence a balance has to be achieved very soon.

Lambert provides the scientific framework upon which evaluations and judgements can be made by those with limited knowledge of nuclear matters, by contrast to Gould and Goldman who present areas of concern which are not based upon proven scientific evidence, but rather the extrapolation of controversial findings well beyond any reasonable level of acceptance. Nevertheless, there are some facts in the fiction — but of course this is

common to many areas of controversy, especially when evaluating man's impact on the environment in relation to technological and social evolution.

In the short to medium term, there are no viable alternatives to the generation of electricity by nuclear means, neither are there any serious technological difficulties involved in the process. To expect a no-effect relation between technology, man and the environment is not reasonable. Overall, the benefits of accepted technology outweigh the disadvantages. It is to be hoped that the nuclear debate will become more open and informed so that the advantages and disadvantages can be properly evaluated. □

E. I. Hamilton is an environmental consultant and Director of Phoenix Research Laboratory, Pengele, Milton Abbot, Dunterton, Tavistock, Devon PL19 0QJ, UK.

Cold fusion and other matters

FOR many years, trenchant observations on the affairs of science in the United States have been provided by the legendary Grant Swinger, director of the mythical Center for the Absorption of Federal Funds. Here Dr Swinger converses with Daniel S. Greenberg of *Science & Government Report*, on 1 June 1989.

Q. How do you see the Bush Administration shaping up in the science-policy area?

A. That's best judged after they take office.

Q. They've been in for over four months.

A. Four months? As long as that. It is difficult to keep track of time in the absence of activity. But I can say that the general atmosphere in the country and in Washington is favourable to innovative approaches.

Q. Example.

A. Cold fusion. An exemplary case in the best tradition of the Center for the Absorption of Federal Funds. If I have any concerns, they're directed at my own institution for not doing it first. Think of it: no publication, just a press conference. And they get \$5 million from the Utah legislature. Jim Fletcher, the ex-chief of NASA, joins up with them, and over a hundred corporations line up and plead for a piece of the action. It's on the covers of *Time* and *Newsweek*, and Congress invites them in to talk about \$25 million. And if there's anything there, no one can find it! This is a triumph. We've worked for years to get the Congress, the press, and the public to this stage.

Q. Very unusual events.

A. Actually, we've pulled this off in medical research many, many times. There's almost nothing that hasn't been reported cured or on the brink of a cure. But this has hardly ever been done in the

physical sciences. The closest thing now going is the search for extra-terrestrial life, but that's never got beyond a small scale.

Q. On cold fusion, there was some scepticism at the outset.

A. That's bound to happen when you try to undercut other researchers in a field. The main opposition came from the hot-fusion tokamak crowd — very innovative people when it comes to protecting their territory. After the first report about cold fusion came out, they advised Congress not to put any money into it until it's proven.

Q. That seems to be a prudent approach.

A. Prudent to wait until it's proven? Hot fusion has been running for 30 years without anything proven. And they're now at \$400 million a year.

Q. They feared competition?

A. It was potentially very embarrassing, very threatening, this tabletop stuff for a mere \$100,000. But do you know the worst of it?

Q. What?

A. The worst of it was that these fusion researchers shouted that they used their own money. As far as I'm concerned, that's unforgivable. They're cannibals. Whether or not cold fusion works is a minor matter. But using their own money. The precedent, I mean —

Q. Please, calm down, Dr Swinger. What are you planning at your centre?

A. We have a number of activities. Inspired by recent events, we're working on an alternative to the superconducting super collider.

Q. What will that be?

A. Table-top particle acceleration. Our motto is: "Fifty-three inches is better than 53 miles. One in each state." The politics are sound, even better than our old proposal for a transcontinental linear accelerator

that would run across a dozen or so states. That would have brought in 24 senators and maybe a hundred congressmen.

Q. Is table-top particle acceleration feasible?

A. We're going about this in the right way to find out. First, we're setting up a task force to plan a workshop preliminary to holding a conference. Next comes a call for papers. Then we'll circulate the proceedings for comment and issue a draft report. A small conference will follow to oversee preparation of the final report. This is the standard way of approaching these things. We'd be criticized if we went about it in any other fashion.

Q. If I may say so, table-top particle acceleration does seem too far-fetched to warrant all that effort.

A. This is an open-ended business. Opportunities can be anywhere. A lot of people thought Star Wars was too far-fetched to get a nickel. But there it is. And what about the manned space station?

Q. What about it?

A. It's terrific. No one knows how much it will cost or what it's supposed to do. But it's going ahead. That's an ideal project, in our view.

Q. What do you foresee as new growth areas?

A. There's a lot of talent out there working up new possibilities. We were worried about the Department of Energy losing that old entrepreneurial spirit, but they reassured us all by cooking up the mapping of the human genome. It's not really their business, but that lit a fire under NIH and they've moved into the genome trade. Of course, it also took a little nudging about the Japanese rushing into this before Congress came across with the money. But bless the Japanese. They've arrived just as the steam was going out of the warnings about how we have to beat the Russians in this or that. What would we do without the Japanese? Without them, superconductivity research would be in the poor house.

Q. What else do you see ahead for growth in science and technology?

A. The greenhouse effect is bound to be a gold mine. And we see a lot of forthcoming activity devoted to agonizing over scientific priorities: conferences, white papers, congressional hearings, and all the usual stuff. It's a small industry all by itself.

Q. In this period of economic challenge, well-chosen scientific priorities are very important for making the best use of valuable scarce resources, aren't they?

A. What?

Q. I was noting the importance of well-chosen scientific priorities.

A. I see. Excuse me, I'm late for a meeting. □

The Grant Swinger Papers, 2nd edn. *Science & Government Report*, 6226 Northwest Station, Washington, DC 20015: 1990. Pp. 40. \$8.95 (\$10.95 overseas orders).

Shifting mosaic

Peter D. Moore

Dynamic Biogeography. By R. Hengeveld. Cambridge University Press: 1990. Pp. 249. £30, \$54.50.

"DYNAMIC" is an interesting word to use in the title of a book. It suggests that the subject may well be perceived by the potential reader as static, if not stagnant, and that the author intends to dispel such an illusion. This is partially true of biogeography. There was certainly a time, perhaps just two decades ago, when biogeography was an essentially descriptive subject, being concerned mainly with the classification of distribution patterns of species and communities, but with little emphasis on the processes involved in the establishment of pattern. But this has changed in more recent times, and one might reasonably expect any modern text on biogeography to be "dynamic".

It is the insertion of the time element that converts a static approach into a dynamic one, and this must be the first test of the claim inherent in the title. Hengeveld certainly passes this test as he sets out to examine both temporal and

spatial variation in biogeographical patterns and to explain underlying causes, emphasizing climatic and human induced factors. One of the main problems of biogeography, however, is what Hengeveld terms the "idiosyncratic properties of species distributions". It is difficult to make general statements on the basis of the wide range of patterns and the spread of causative factors found among plant and animal species. The detection of pattern within the patterns clearly demands sophisticated analytical techniques, and the first part of the book is concerned with the application of multivariate statistics to the classification and ordination of species-distribution data. This initial process in biogeographical methodology provides, in Hengeveld's view, an efficient system for hypothesis generation, such hypotheses usually relating either to the past history of the taxa, or to their ecological demands and limitations. But the two types cannot be clearly separated, for the physical and biological environment is itself not static, so ecological demands simply place limits on species distribution within the ever-rolling historical framework.

The logical progression of this line of thought would take us into a consideration of changes in the ranges of selected taxa

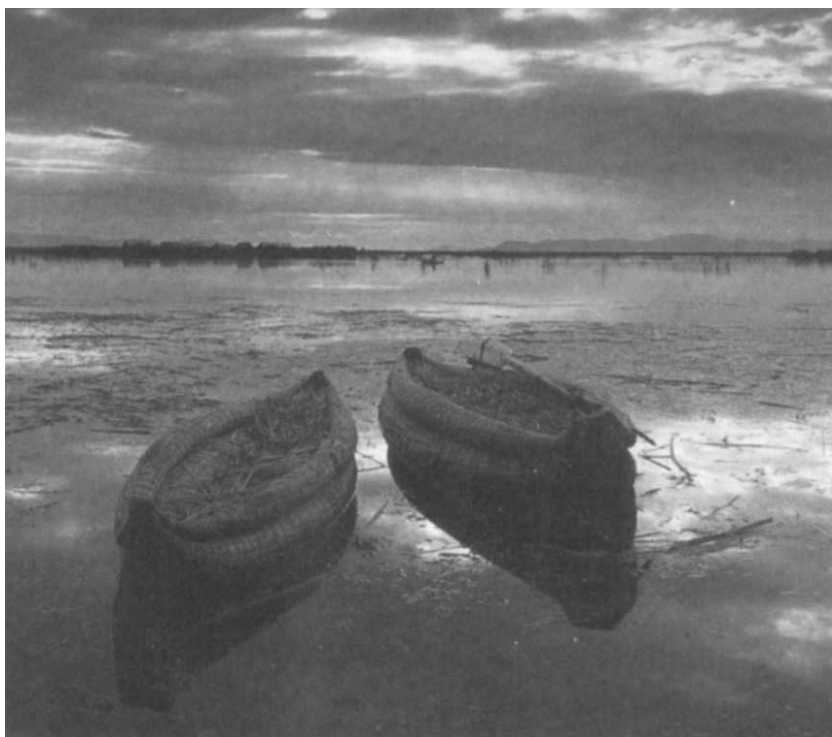
over various timescales, but the author chooses to leave this development until later in the book and to insert a section on geographical trends in species richness and other biotic features. If one reads the book sequentially, then this tends to disrupt a well-constructed argument — but the topic is one of considerable biological interest and certainly demands coverage. There are some biogeographical generalizations that are supra-taxonomic, such as climatic relationships to diversity, vegetation architecture, life form, leaf form and so on, that can assist in an understanding of the spatial arrangements of the world's biota. Hengeveld tackles this section of the book by choosing specific examples to illustrate the general development of biogeographical thought in these fields, such as gradients of bird richness in North America, species "nesting" and its relationship to refugia hypotheses, C3/C4 photosynthetic strategies and climate, and many others.

The author finally returns to the theme of individual species' ranges and their analysis. He considers general features of population response curves to environmental variables and illustrates these with examples in which species boundaries can be related to climatic factors. On its own, however, such an approach might be regarded as static rather than dynamic, so Hengeveld concentrates on species for which there is adequate historical information from the fossil record so that the time factor can be incorporated into the model. The examples he uses, like *Tilia cordata*, are selected to illustrate the potential of this technique. But such relationships are rarely simple and neat, and in the final section of the book Hengeveld considers some of the complicating factors, such as the stochastic impact of weather and the lag that may exist between environmental change and species response.

The book provides a realistic account of what has been achieved by modern biogeography. But it also leaves me with the impression that the future of biogeographical development is largely in the hands of the ecophysiologicalist and the population geneticist, with some help from the statistician. I would say that the taxonomist and the palaeoecologist still have important parts to play in the assembling of data upon which testable hypotheses can be established.

This is a stimulating book, well written and arranged. There is a refreshing, logical line of argument that underlies the text and which leads the reader painlessly through the morass of information that typifies biogeography. It well justifies its claim to be dynamic. □

Peter D. Moore is in the Division of Biosphere Sciences, King's College London, Campden Hill Road, London W8 7AH, UK.



Whatever you do, wherever you go, plants will have made some contribution — this is the message of *Plants for People* by Anna Lewington. The volume presents both well known and not so well known uses of plants; there are details of those that protect, cure, transport (reed boats above), entertain and the better known examples of those that clothe and feed us. The book, well researched and presented, illustrates almost all examples in full colour. Interesting information gleaned from the pages includes the story of the fine cloth made from pineapple leaves, the bamboo used for scaffolding and the anaesthetics from the Andes. Published by The Natural History Museum, London. Price is £19.95. □

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New paperbacks

■ Recently reissued as a paperback by Freeman is Steven Weinberg's *The Discovery of Subatomic Particles*, originally published in 1983 as part of the Scientific American Library series. Price is £11.95.

■ *Ancient Forests of the Pacific Northwest* is edited by Elliot A. Norse under the auspices of the Wilderness Society. The essays in the book cover the "grandeur, complexity, diversity and impending destruction of a fragile and vital ecosystem". Publisher is Island Press, price is \$19.95.

■ Two new volumes in the Laser Science and Technology International Handbook series are *Photobiology of Low-Power Laser Therapy* by T. I. Karu, price \$54, and *Refractive Non-linearity of Wide-Band Semiconductors and Applications* by A. A. Borhch, M. Brodin and V. Volkov, price \$42. The first few volumes in the series were reviewed by Peter Knight in *Nature* **344**, 501; 1990. Publisher is Harwood.

■ The latest volume from the Panos Institute is *Miracle or Menace? Biotechnology and the Third World* by Robert Walgate, who used to be *Nature's* European correspondent. The volume explains the science behind, and examines the implications of, new developments in biotechnology. Price is £6.95.

■ New from the University of Chicago Press is *Frozen Fauna of the Mammoth Steppe* by R. Dale Guthrie. The book is about the mummified mammals frozen since the ice age, in particular the 36,000-year-old bison called "Blue Babe". Price is £13.50, \$19.50. (Also available in hardback at £31.95, \$45.95.) □

Trucking on

Paul Calvert

The American Synthetic Rubber Research Program. By Peter J. T. Morris. University of Pennsylvania Press: 1989. Pp. 191. \$34.95, £33.20.

AT THE outset of the Second World War tyres were mainly made of natural rubber from Indonesia and Malaysia. To avoid a loss of supplies, both Germany and the United States put a huge effort into building a synthetic rubber industry. The best available rubber was a styrene-butadiene copolymer prepared by emulsion polymerization. This was first developed in

search progress into incremental improvements, large improvements and breakthroughs. Incremental improvements in processes came continuously and were shared between the companies, but probably would have come anyway. Large improvements could mean a significant competitive advantage to a company so each one tried to keep these outside the programme and the pool of shared information. Breakthroughs can be very expensive and can upset a smoothly running bureaucracy and thus are to be avoided if possible. There really were none of these in the course of the programme.

The bulk of the \$7 million university funding went to Illinois, Chicago, Minnesota and Cornell. Marvel's group at

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Searching for new polymers — no alternative in the haystack

Germany in 1929 and was adequate for car tyres but not good enough for truck tyres. There was no way to synthesize "natural rubber", *cis*-polyisoprene. From 1942 to 1956 the US Government spent \$56 million on a well-organized research effort to ensure a domestic supply of rubber for car and truck tyres. The goals were then to set up efficient production plants, to improve the properties of the rubber produced and preferably to replace it with a better synthetic rubber.

Morris has interviewed many of the participants in the scheme and asks whether the research and development programme was a success. His answer is a qualified "no". The overall production programme was a success in that \$700 million was invested in 51 plants to produce 720,000 tons of rubber a year by 1945, but the research produced no major new rubber and when synthetic *cis*-polyisoprene finally came it was too expensive. The main participating companies were Goodyear, Goodrich, US Rubber and Firestone with Phillips Petroleum and General Tire on the sidelines.

It is fascinating to follow Morris to his conclusions. One reads a section thinking "Well, this aspect was not a success, but he has forgotten such-and-such a spin-off" and then sees that argument demolished in the next chapter. Morris divides re-

Illinois put a large effort into developing more than 100 new rubbers but none of them were better than butadiene-styrene. It is a characteristic of materials research that the first round of work often produces a material which is never bettered in subsequent projects. Flory at Cornell did a great deal of good polymer science but seems to have been regarded as extraneous to the main programme. The late 1940s and 1950s were a time of very rapid growth in polymer science and in the plastics industry. Morris argues that the academic contribution to progress by the rubber industry was small. There was mistrust of the universities by the companies. On the academic side, there was little knowledge of industrial problems and a lack of appreciation of short industrial timescales.

Many of the participants in the programme think of it as a great success. It is a bit unfair to criticize them for not finding an alternative rubber for truck tyres, for it is clear that there really wasn't one. The haystack had no needle in it.

Morris is not a polymer scientist, but there are few places where this really shows. An appendix summarizes polymer chemistry for the uninitiated. □

Paul Calvert is in the Department of Materials Science and Engineering, University of Arizona, Tucson, Arizona 85712, USA.

Activators and targets

Mark Ptashne & Alexander A. F. Gann

Proteins that activate genes are quite disparate in character; in particular, some work 'universally' and others do not. A simple model can accommodate most of the recently published results.

SEVERAL papers recently published in *Nature* and elsewhere address certain details of the mechanism by which eukaryotic transcriptional activators work, and reach conclusions that are in apparent conflict. We shall briefly review the essential conclusions of these papers to see whether they can be reconciled. We begin with an outline of the broad ideas and findings that constitute the background of the new work.

According to our current picture, a DNA-bound transcriptional activator interacts with some component of the transcription machinery, bringing it to the DNA and/or changing its conformation on the DNA (ref. 1). This event leads to, or completes, the formation near the transcription start site of a 'pre-initiation complex' that contains several proteins in addition to RNA polymerase itself, including TFIIA, B, D, E and F (ref. 2).

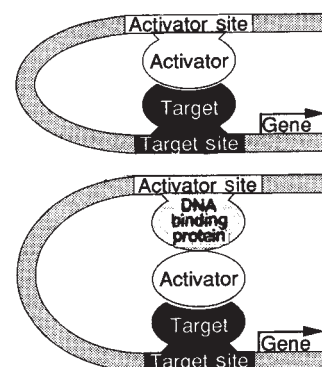
We know of two classes of activators: members of one work 'universally', whereas members of the other work only in certain cells. An example from the first class is the yeast transcriptional activator GAL4: when introduced into any of a wide array of eukaryotic cells, GAL4 activates transcription of a gene bearing GAL4 sites nearby³⁻⁶. According to the simple model shown in Fig. 1, the key to the universal action of GAL4 is that it bears two functions on a single peptide: a DNA-binding surface and an 'acidic activating region' that interacts with a target protein the relevant surface of which must be conserved among eukaryotes. Each member of the second class of activators, by contrast, bears just one of the two required functions and therefore can work only in cells bearing molecules that provide the missing function. We consider as examples the mammalian DNA-binding protein Oct1 (ref. 7) and the herpes virus protein VP16 (refs 8-11). As shown in Fig. 1, VP16, which bears an acidic activating region, is unable to bind to DNA directly. It can, however, bind to a complex of DNA-bound proteins that includes Oct1, which itself lacks an acidic activating region¹². Thus VP16 will activate transcription only in cells containing Oct 1, and Oct 1 will activate genes only in cells containing VP16. When VP16 is fused to a DNA-binding fragment of GAL4, the fusion protein works as does GAL4 itself¹³. According to this model, then, the 'non-universal' activators can be understood in the same framework as the 'universal' ones. It may be helpful for what follows to bear in mind that 'activator' is an operational definition, applied to any transcription factor that activates specific genes. Thus the proteins GAL4, Oct1 and VP16 are all called activators, despite their dissimilarities.

The new papers we consider address two questions: first, what is the target protein seen by acidic activating regions? And, second, can we fit into the picture outlined above two additional non-acidic regulators that work only in certain cell types: SP1, a mammalian DNA-binding protein¹⁴, and Ela, an adenovirus protein that does not bind DNA¹⁵? Both of these proteins activate in mammalian but not in yeast cells, a result that holds for fusion proteins comprising SP1 or the Ela activating region (as defined in mammalian cells) attached to DNA-binding fragments of GAL4 (G. Gill, E. Pascal, R. Tjian and M. P., unpublished; J. Lillie and M. Green, personal communication; ref. 16). We begin by asking what is the target seen by acidic activating regions?

VP16 and TFIID

Stringer *et al.*¹⁷ argue that the activating region of VP16 interacts directly with the protein TFIID. They report that an affinity column bearing VP16 retains a protein — or proteins — that restores activity to a mammalian cell extract that has been depleted of TFIID activity by heat inactivation. Moreover, this column selectively retains yeast TFIID expressed from a cloned gene in bacteria¹⁷. These results are made more compelling by the claim that there is a good correlation between the strengths of various VP16 mutants, as assayed by their abilities to activate transcription in mammalian cells, and their

FIG. 1 Gene activation by different kinds of activators. *a*, The activator bears on one peptide a DNA-binding surface and an acidic activating region. The activator, bound at its cognate site, brings to the DNA and/or changes the conformation of a 'universally' conserved target protein that binds near the 5' end of the gene, the intervening DNA looping out to accommodate the reaction. GAL4 is an example of such an activator¹. *b*, An acidic activating region and a DNA-binding surface are carried on separate molecules, and both molecules are required for activation. Oct1-VP16 is an example¹²⁻¹⁴.



affinities for TFIID as measured in the column experiment (ref. 17 and C. J. Ingles, M. Shales, D. Cress, S. Triezenberg and J. Greenblatt, personal communication). The result is so important that we look forward to further controls showing, for example, that acidic activators do not interact with other general transcription factors, and that at least some mutants of TFIID that cannot respond to acidic activators in a transcription assay also cannot interact with the acidic activating regions as measured directly.

The idea that TFIID is the target of acidic activating regions is attractive: binding of TFIID is known to precede binding of the other known transcription factors and to 'commit' the template to being transcribed once these have been added². Previous experiments had shown that various activators change the footprint made near the TATA sequence in a partially purified TFIID preparation (a TFIID fraction) from mammalian cells^{18,19}.

If Stringer *et al.*¹⁷ are right, the phenomenon of universal activation requires that all the TFIID proteins found in various eukaryotes can interact with acidic activating regions. This proposition can be tested now that TFIID genes have been cloned from HeLa cells^{20,21}, *Drosophila*²² and *Arabidopsis*²³ as well as yeast²⁴⁻²⁶. As in yeast, each of these organisms evidently has only a single TFIID gene, with the exception of *Arabidopsis*, which has two²³. These genes are similar throughout their carboxy-terminal 180 amino acids, but differ in their amino termini (Fig. 2). In particular, those of human and *Drosophila* each have a stretch of about 160 amino acids absent from the other molecules. The yeast TFIID has approximately 60 amino acids at its N terminus that bear no similarity to sequences in *Drosophila* or human TFIID.

A missing component

The chromatographic fraction termed TFIID (that which includes the TATA binding protein) has never been purified to homogeneity from extracts of mammalian cells, and it was not unreasonable to expect that this fraction includes more than one component. There is now strong evidence for this. Mammalian TFIID, synthesized in

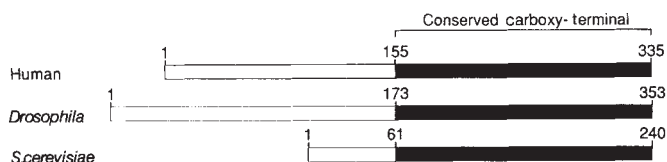


FIG. 2 The products of various cloned TFIID genes²⁰⁻²⁶. The molecules are similar, but not identical, over approximately 180 carboxyl-terminal amino acids. The amino termini bear distinct amino-terminal extensions.

and purified from bacteria, restores basal level transcription to a mammalian extract lacking the TFIID fraction, but does not restore the ability to respond to acidic activators such as GAL4-VP16, or to SP1. In an important control for this result, Peterson *et al.*²⁰ show that an extract treated with heat to destroy its transcriptional activity can be restored to full inducibility by the addition of cloned human TFIID; this result confirms that heat specifically inactivates TFIID, and that the recombinant protein is functional (ref. 20; R. Tjian, personal communication).

These results strongly suggest that the TFIID fraction of the mammalian cell extracts contains some component or components, in addition to TFIID itself (as now defined by the cloned gene), that is necessary for response to any activator. The fact that TFIID as partially purified from mammalian cells behaves as a larger protein than the cloned TFIID²⁷ suggests that this new component in the mammalian case at least may be bound to TFIID. Evidently this interaction has some species specificity: addition of excess yeast TFIID to a mammalian or *Drosophila* extract elicits a basal level of transcription that can be activated neither by GAL4-VP16 (R. Tjian, personal communication) nor by SP1²⁸. Presumably, in this experiment, the added TFIID competes with the endogenous TFIID for binding to the TATA sequence and, once bound, is unable to respond to activators.

If we accept the results of Stringer *et al.*¹⁷, the extra component(s) required for activation is probably not an intermediary between an acidic activating region and TFIID: it is more likely to be a factor required for some step in activation that follows activator-TFIID interaction (see for example Fig. 3). Kelleher *et al.*²⁹ and Berger *et al.*³⁰, however, argue to the contrary, proposing the existence of an intermediary between an acidic activating region and TFIID. Their arguments are indirect and rely on "squenching" experiments^{1,31} performed *in vitro*. The idea behind "squenching" is that the putative interaction between the activator and the target, as shown in Fig. 1, might also occur off DNA. Thus an activator bearing a strong activating region and present at artificially high concentrations could sequester the target and thereby inhibit, or "squench", the expression of other genes (Fig. 4). Indeed, expression of a series of GAL4-derived activators at high concentrations in yeast inhibited expression of a heterologous gene, and the degree of inhibition was roughly proportional to the strength of the overexpressed activating regions³¹. Another example is seen with VP16, whose activating region is unusually potent; at high concentrations, VP16 is a particularly strong squencher in mammalian cells^{8,32}.

In the squenching experiments of Kelleher *et al.*²⁹, GAL4-VP16 is added to a yeast extract containing two added genes: one bears

GAL4 binding sites upstream and the other dA.dT sequences that presumably bind an endogenous activator. Moderate levels of GAL4-VP16 activate the former as they inhibit the latter (squenching), and high levels of GAL4-VP16 inhibit both (squenching and self-squenching). Kelleher *et al.*²⁹ then find that this inhibition is not relieved by addition either of TFIID or of polymerase, but is relieved by addition of some other fraction of the yeast extract. The authors surmise that this fraction includes an adaptor that bridges TFIID and the acidic activator. Their results are, however, equally consistent (as the authors note in passing) with the idea that the additional component, rather than being an intermediary, is required for some essential step in activation that follows activator-TFIID interaction. Should that third component be limiting, squenching might be relieved by addition of more of that component, but the result would not reveal the species contacted by the activator.

Similar experiments by Berger *et al.*³⁰ are more difficult to reconcile with those of Stringer *et al.*¹⁷. Berger *et al.*³⁰ also demonstrate squenching of a heterologous promoter by GAL4-VP16 in yeast extracts. But if an excess of a short oligonucleotide bearing a single GAL4 binding site is included, activated transcription is squelched but basal level is not. Berger *et al.* interpret these results as follows. In the absence of the competing oligonucleotide, non-specific DNA binding fosters complete squenching; they imagine the formation of a three-component complex consisting of an activator, an intermediary, and TFIID, with both the activator and TFIID making non-specific contacts with DNA. By contrast, when the activator is bound to the short oligonucleotide, the hypothetical intermediary is sequestered but TFIID is not, and thus basal level transcription is undiminished. The authors' interpretation of this experiment would exclude the possibility that VP16 interacts with TFIID, and thus contradicts the results of Stringer *et al.*¹⁷.

The following experiment, to our knowledge not yet done, might clarify the matter. Squenching by VP16 could be tested in a mammalian cell extract in which cloned TFIID replaces the TFIID fraction; as we have noted, only basal level transcription is observed in such a system. Stringer *et al.*¹⁷ would predict that, even though VP16 cannot activate in this system, it would nevertheless squelch the basal level transcription. Were squenching not observed, the simplest explanation would be that there is an adaptor, as predicted by Berger *et al.*³⁰, and that that adaptor had been removed as part of the TFIID fraction.

The results described so far (with one exception³⁰) are consistent with two propositions, as shown in Fig. 3. First, acidic activators interact directly with TFIID and second, another component(s)

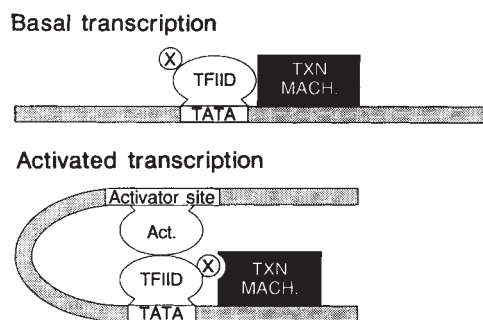


FIG. 3 An auxiliary protein required for activation. As described in the text, the acidic activator VP16 interacts specifically with cloned TFIID¹⁷; GAL4-VP16, however, will not activate transcription in a mammalian extract in which the TFIID fraction has been replaced with cloned TFIID²⁰. To rationalize these results we imagine a second protein — termed X — that is required for TFIID's response to the activator. Protein X need not be contacted by the activator, but might nevertheless be bound to TFIID in the absence of the activator (as depicted in the figure). Alternatively, X might bind to TFIID only after the activator has caused a conformational change in TFIID. The response of the transcriptional machinery to the activator may involve a change analogous to the transition from a "closed" to an "open" complex elicited by an activator (NTRC) working on *E. coli* RNA polymerase bearing $\sigma 54$ (refs 37,38). Another interesting analogy is activation of phage T4 late gene expression. The promoters of T4 late genes are recognized by RNA polymerase bearing the T4 encoded $\sigma 55$. Transcription by this polymerase is activated by a complex of the products of the T4 genes 44, 62 and 45, but only if the polymerase also bears a further protein, gp33, itself not necessary for promoter recognition nor, at least under some conditions, for basal level transcription³⁹. The role of gp33 is unknown.

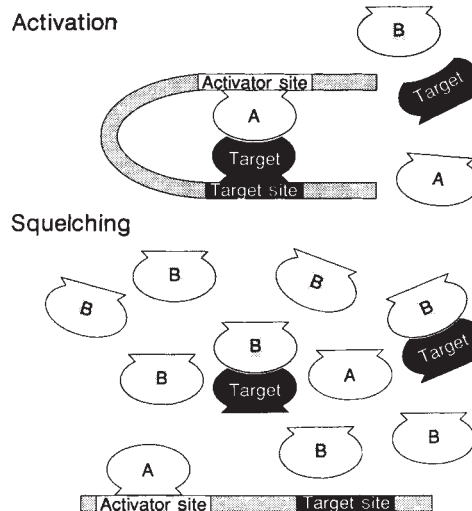
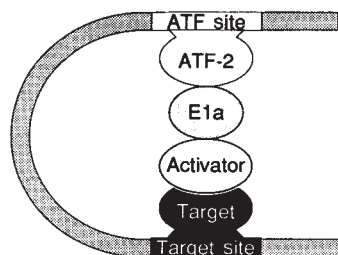


FIG. 4 Squenching. High concentration of activator B sequesters the target protein also seen by activator A, thereby inhibiting activation by A³¹. At very high concentrations activation by A will also be inhibited (self-squenching). Note two features implied by this picture: first, the sequestered protein need not be the target as defined in Fig. 1. Thus, for example, overexpression of E1a presumably sequesters an intermediary protein that itself might bear an activating region that interacts with the target of Fig. 1 (ref. 32). Second, the factor sequestered by the activator need not be a single species and, assuming that any of the peptides in the complex might be limiting, squenching could be relieved by addition of that peptide.

FIG. 5 A model for activation by E1a. E1a recognizes, with one surface, a DNA-bound ATF-2 (ref. 34), and with another surface, a protein bearing an acidic activating region. Note that although there is evidence suggesting that E1a contacts an intermediary protein, there is no evidence that that protein bears an acidic activating region³².



must interact with the TFIID-activator complex for a full response to the activator. Before attempts to apply this model to the mammalian activators E1a and SP1, we summarize results of experiments involving nucleosome formation *in vitro* that are consistent with the model.

Workman *et al.*³³ found that when a nucleosome assembly system is added along with the template DNA to a mammalian cell extract, the basal level transcription observed in the absence of the nucleosomes is blocked; presumably nucleosome formation competes with and excludes binding of some transcription factor (for example TFIID). It has now been reported that inclusion of GAL4-VP16 in the initial mix elicits a high level of transcription if the template bears GAL4 binding sites upstream (J. L. Workman, I. C. A. Taylor and R. E. Kingston, manuscript in preparation); evidently the activator helps some transcription factor (such as TFIID) compete with nucleosome formation for DNA binding. When this experiment is repeated, using an extract in which cloned yeast TFIID replaces the TFIID fraction, basal level transcription is observed. Workman and co-workers' interpretation is that, either directly or indirectly, GAL4-VP16 helps yeast TFIID to compete for binding with nucleosomes; only basal level transcription is observed in this case, however, because a subsequent, perhaps species specific, interaction is required for full activation.

E1a and SP1

M. Green and colleagues show convincingly³⁴ that E1a binds to the DNA-bound protein ATF-2, suggesting that E1a works in a fashion analogous to VP16. But various considerations lead them to suggest that E1a does not bear an acidic activating region, and they interpret experiments involving squelching in mammalian cells as arguing that E1a works by a mechanism that involves a simple extension of model *b* in Fig. 1³². They propose that E1a, attached by one surface to DNA-bound ATF-2, in turn attaches by another surface to a third component; that component could bear an acidic activating region (Fig. 5). Their experiments can be summarized as follows: expression of a gene activated by any of several GAL4-derived activators bearing acidic activating regions is squelched by high levels of VP16, but not by high levels of E1a. By contrast, expression of a gene activated by the fusion protein LexA-E1a (which binds to LexA operators) is squelched by high levels of either E1a or VP16. These results are consistent with the idea that E1a is an intermediary that contacts an activator protein that, in turn, contacts the target seen

by VP16. Note that, if the argument is correct, the activator protein contacted by E1a cannot be an essential component of the transcription machinery, because high levels of E1a do not squelch VP16-activated genes.

Turning now to SP1, we might imagine three mechanisms for how this non-acidic DNA-binding protein activates. First, it might, like Oct1, bind an activator that bears an acidic activating region that interacts with TFIID as described by Stringer *et al.*¹⁷. Second, SP1 might, directly or via an intermediary, contact a part of TFIID different from that recognized by the acidic activators. Third, it might contact, directly or indirectly, some component of the transcription machinery other than TFIID. At the moment there is no compelling basis for a choice between these models. Pugh *et al.*²⁸ show that mammalian recombinant TFIID is unable to substitute for the TFIID fraction in supporting a response to SP1, a result they interpret as indicating the need for an intermediary between SP1 and the TFIID polypeptide. But as we have noted, the same result holds for acidic activators and, assuming that Stringer *et al.*¹⁷ are right, the missing component(s) could be required for activation without interacting with the activating region (Fig. 3). The sequences of the various cloned TFIIDs could hint at a version of the second model: SP1, which does not activate in yeast, might recognize the amino-terminal extension found on human and *Drosophila* TFIID that is missing from yeast TFIID. Unfortunately, deletion of that part of human TFIID, which abolishes the response to SP1, also abolishes the response to an acidic activator³⁰, and so the question remains undecided.

Conclusion

It is possible (*pace* Berger *et al.*³⁰) that the following simple model holds: all activators exert their effects by interacting with TFIID: acidic activators work directly, and hence universally, and others (Oct1, SP1, E1a, for example) do so via intermediaries bearing acidic activating regions. Even if the experiment of Stringer *et al.*¹⁷ were to turn out to be misleading, and acidic activators were to recognize some target other than TFIID, it would still be possible that all activators work through that target. But as the models for the possible action of SP1 suggest, there are other, more elaborate, possibilities. In any event, at least one aspect of the simple model is puzzling: it is now well established that heterologous acidic activators can work synergistically to activate transcription, and recent experiments support the idea that their effect is caused by several acidic activating regions simultaneously touching the target^{35,36}. If TFIID is indeed the target of acidic activators, we will have to explain how these multiple activating regions can simultaneously touch the rather small (*M*_r 38,000) TFIID protein.

Note added in proof: Cheng Kao *et al.* (*Science* **248**, 1646–1650, 1990) also report the cloning of human TFIID. They find that when their cloned mammalian TFIID, or cloned yeast TFIID, replaces the TFIID fraction in a mammalian extract, transcription is activated by a protein (presumably SP1) that binds to GC-rich sequences near the gene. This result is in striking contrast to results cited in the text^{20,28}. □

Mark Ptashne and Alexander A. F. Gann are in the Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, Massachusetts 02138, USA.

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Oxygen-defect flux pinning, anomalous magnetization and intra-grain granularity in $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$

M. Daeumling*, J. M. Seuntjens & D. C. Larbalestier

Applied Superconductivity Center, University of Wisconsin, Madison, Wisconsin 53706, USA

The anomalous magnetization characteristic, intra-grain granularity and the magnitude of the flux pinning in single crystals of $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ are shown to be intimately linked. As the nominal oxygen deficiency δ decreases towards zero, the flux pinning declines and the crystals lose their explicitly granular signature. As δ is seldom specifically controlled at levels below ~ 0.05 in even the most carefully made materials, small oxygen deficiencies may contribute significantly to both the flux pinning and the weak-link properties.

THE flux pinning and granular properties of high- T_c superconductors continue to be a perplexing problem. Crystallographic grain boundaries are strongly implicated as the decoupling sites in these materials¹ and thus single crystals should be important for the study of the basic pinning mechanisms. It has been shown, however, that single crystals separated from bulk polycrystalline samples can also become granular (that is, internally subdivided)^{2,3}. Also, a study of a large single crystal of $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ with a low T_c of 82 K showed it to be granular⁴. On the other hand, it has also been reported that individual crystals are fully coupled^{5,6}. Thus there is no general agreement on whether intra-grain weak links are generally present and measurements on 'good' single crystals over a wide range of temperature and field are needed to resolve this important issue. Here we describe such measurements.

Magnetization characteristics

We used two crystals of $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ grown from CuO-rich flux^{7,8} and one grown by a liquid-phase process⁹. Details are given in Table 1. The first crystal (FG1) was selected because of its high T_c , the sharpness of its a.c. inductive transition and its complete Meissner flux exclusion in fields < 0.1 mT, which indicate that this crystal should be considered 'good' by present standards. Our characterization confirmed the very sharp transition in the zero-field a.c. susceptibility (T_c midpoint 93.5 K, ΔT_c (10%, 90% signal) < 1 K). But when weak d.c. magnetic fields were applied, the transition was found to occur over several degrees. In all but the weakest magnetic fields (~ 1 mT), we observed smeared double transitions, indicative of granularity. Striking effects were also seen in magnetization measurements made with a vibrating-sample magnetometer. Figure 1 shows the magnetization loop at 70 K (for an applied field H_a parallel to the c axis, as was the case for all measurements reported here); an anomalous and large second peak was observed at ~ 4.5 T, the magnitude of the second peak being about twice the zero-field peak. In Fig. 2, the magnetization data for the range 10–80 K and up to ~ 8 T is reduced to J_c using the standard expression $J_c = \Delta M / (2r)$, where ΔM is the width of the hysteresis loop and r the effective crystal radius ($r = \frac{1}{2}l_1(1 - l_1/3l_2)$, where l_1 is the shorter dimension, perpendicular to the field). The double peak is observed from 30–80 K, although the anomaly is particularly evident above 50 K. Observation of the second peak requires measurements over a wide range of T and H and therefore the second peak is frequently not seen. The minimum in M or J_c , however, is generally visible but almost invariably ignored^{10–13}. Thus, the effect is very general in twinned and untwinned crystals, which are normally accepted as being 'good', as well as in samples recognized to be of lower quality⁴. Further insight is provided by the data of Fig. 3, which shows the magnetization at fields of 0, 1 and 4 T at 80 K for three pieces of the crystal (FG1) after subdivision. At low fields (0–1 T), M is proportional to size, as is expected for a coupled crystal, whereas at higher fields (4 T), well above the second peak, M is independent of r . The latter behaviour is characteristic of a granular crystal. At low temperatures, however, M (0–4 T) was always proportional to size¹⁴.

One of the variables that these results stimulated us to explore was the effect of small oxygen deficiencies δ on the superconducting properties. Oxygen diffusion coefficients in $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ are rather uncertain^{15,16} and the oxygen content of single crystals, weighing only a few hundred micrograms, cannot be explicitly checked by the techniques applicable for bulk samples. A common oxygenation applied to flux-grown crystals is 240 h at 420 °C^{7,8} for which the equilibrium value of δ would be ~ 0.04 (ref. 17). In zero-field measurements¹⁸, T_c has been shown to be insensitive to δ for $\delta \leq 0.1$, but this result has not been checked in a non-zero field. Thus the variation of the magnetization properties with oxygenation was the focus of the second part of the experiment. We used two new crystals—one, FG2, was grown in a similar manner to the previous

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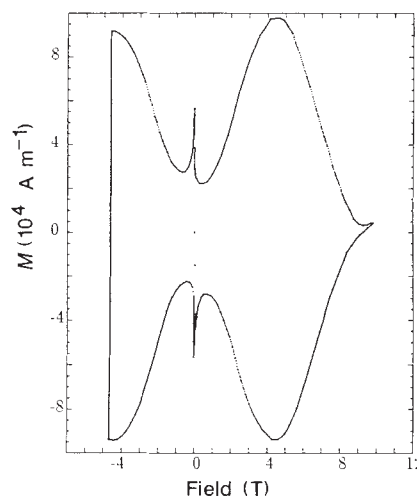


FIG. 1 Magnetization loop of flux-grown crystal 1 at 70 K.

* Present address: IBM, T. J. Watson Research Center, Yorktown Heights, New York 10598, USA.

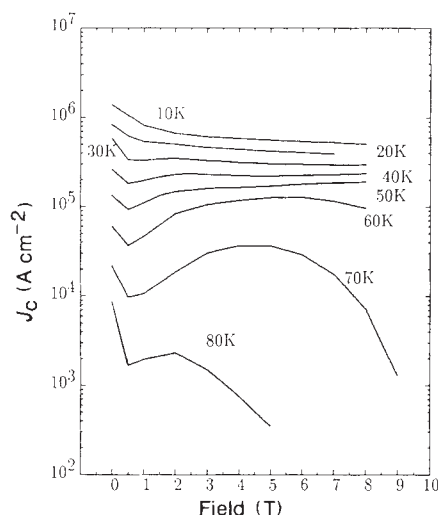


FIG. 2 Critical current density of flux-grown crystal 1 derived from the magnetization loops using the sample size as the scaling radius.

crystal^{7,8}. Like FG1, it was extensively twinned but had no observable second phase. The second sample was made by a modified liquid-phase processing (LPP) route⁹. A highly textured, multi-domain sample composed of about 15 crystals stacked on top of one another was cut from a larger polycrystalline mass. The microstructure of the individual grains contained ~20% of Y_2BaCuO_5 ('211') phase of no obvious preferred orientation. The boundaries between the crystallites were a - b planes, for which the misorientation was very small (the 006-plane rocking curve had a full width at half maximum of $\sim 0.8^\circ$). The a and b directions of neighbouring layers in the crystal also seemed to be very well aligned. Thus, in the direction for which we made measurements (applied field parallel to the c axis), there are no crystallographic grain-boundary barriers to current flow and from an electromagnetic point of view the sample can be considered to be a 'single crystal'.

Initially we equilibrated the specimens at 600 °C for 100 h in 1 atm of flowing oxygen, followed by 100 h at 525 °C. After this treatment, T_c was ≤ 80 K. Here we report data taken after subsequent oxygenation, for which T_c was ≥ 90 K. Derived J_c values for the crystals are shown in Fig. 4. We note several features—first, in both samples the derived J_c decreases with further oxygenation. Thus decreasing δ does reduce the flux pinning. Second, as the oxygenation improves, the magnetization anomaly tends to disappear. This was particularly true for the LPP crystal, for which the anomaly was still visible after the shortest oxygenation (100 h at 450 °C). The anomaly of the FG crystal, however, did not disappear even after >900 h of oxygenation at ≤ 450 °C. Third, the J_c of the flux-grown second-phase-free single crystal exceeds that of the LPP sample, which contains ~20% of 211 phase, over a considerable range of the magnetic field, whether measured at high (70 K) or low (10 K) temperature. It is therefore unlikely that the large (2–10 μm diameter) second-phase particles of the LPP sample are contributing significantly to the flux pinning.

The intra-grain granularity was again examined by subdividing the crystals after their penultimate oxygenation (200 h with slow cooling from 400 to 350 °C). The anomaly for the FG2 crystal was still very clear— J_c rises to a distinct peak at ~ 2 T at 70 K, but at 50 K the maximum was smeared out in the range 2–8 T. For the LPP crystal, however, no such anomaly was observed after the oxygenation exceeded 100 h at 450 °C. The size-scaling results of Fig. 5 for the LPP and FG2 crystals are also very different. For the LPP crystal, $\Delta M(H)$ is proportional to r at both 10 K and 70 K, whereas for the FG2 crystal, the

TABLE 1 Details of crystals

Crystal	Source	Size*	Comments
FG1	IBM	~ 0.85 mm radius $\times 42 \mu\text{m}^\dagger$	Flux grown, single crystal
FG2	NIST‡	1.16×0.90 mm $\times 62 \mu\text{m}^\dagger$	Flux grown, single crystal
LPP	UW§	2.2×1.8 mm $\times 65 \mu\text{m}^\dagger$	Liquid-phase process, multi-domain crystal

* Before breaking.

† Thickness in the c direction.

‡ National Institute of Science and Technology.

§ University of Wisconsin.

behaviour is more complex. At 10 K, no anomaly is seen ($M \propto r$ for $H = 1$ T, 4 T). This is also the case at 70 K for a magnetic field of 1 T, which lies below the $\Delta M(H)$ peak at that temperature. At 70 K and for magnetic fields of 4 T and 6 T, M becomes independent of size. Thus, evidently granular behaviour is visible for crystal FG2 beyond the second peak in the magnetization curve, just as it was for FG1 (Fig. 3).

Discussion

These data are all consistent with the proposal that oxygen defects act as significant pinning centres. In the absence of a magnetic field, proximity-effect coupling between the oxygen-rich and oxygen-deficient regions maintains a sharp zero-field T_c value at a high value^{18,19}. Oxygen-deficient regions should have a markedly lower $T_c(H)$ in a non-zero field, however, and can broaden the T_c transition as the field increases. The elementary pinning force per defect ($\sim \frac{1}{2}\mu_0\Delta H_c^2 V_d$ where ΔH_c is the critical-field difference between matrix and defect and V_d is the fluxon core volume intercepted by the defect) increases as the field suppresses superconductivity in the oxygen-deficient region, causing the magnetization to rise steeply beyond the minimum in the M - H curve (Fig. 1). Similar, but less marked behaviour, has been observed for Sn precipitates in a Pb-In-Sn alloy²⁰. Here, however, we have shown that the flux pinning and the granular properties are linked. Evidently there are enough oxygen-deficient regions^{21–23} to link up and provide internal barriers, rendering the crystal granular. The steep fall in the magnetization hysteresis (Fig. 1) beyond the second peak is then at least partly due to a progressive reduction in the scaling radius as the field increases. Plots of the derived J_c (such as Figs 2, 4) therefore become unreliable when the scaling radius is ill defined, as it evidently is at higher fields for the flux-grown crystals. As expected, M increases at higher fields for specimens with larger oxygen contents, reflecting the better size scaling.

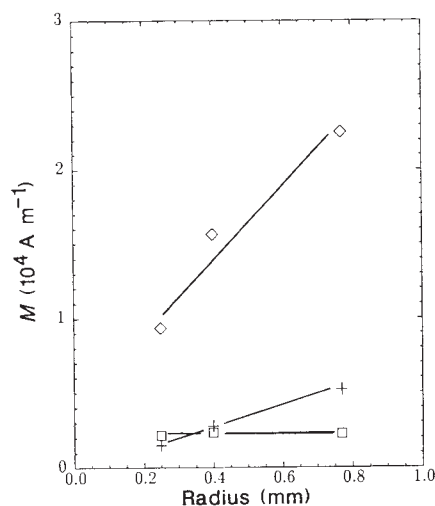


FIG. 3 Magnetization hysteresis plotted against radius for flux-grown crystal 1 at 80 K. $\diamond$, 0 T; +, 1 T; $\square$, 4 T.

An important issue raised by our data is the large magnitude of J_c in the crystals. In optimized NbTi with 47 wt% Ti, where very strong flux pinning exists, 20 vol% of nanometre-scale α -Ti precipitation^{24,25} leads to J_c values of the order of 10^{-2} of the depairing current density, J_d , roughly the same as for these crystals. But, as already discussed, the second-phase precipitates of the LPP crystal do not provide strong pinning. Twins must also be considered as pinning sites. Although there is abundant evidence to show that twins pin flux¹⁰⁻¹², there is no consensus on the relative strength of twin pinning compared to other sources of pinning. A recent study¹² of the same crystal in both the twinned and untwinned state showed that the residual pinning of the untwinned state was, in general, significantly larger than the pinning produced by the twins (except at 10 K for H parallel to the a - b plane)¹². These studies are consistent with the conclusion that there is strong residual pinning, whatever the twin or second-phase density. We therefore conclude that small oxygen deficiencies have a significant pinning role. The average distance d between unordered O vacancies is $\sim a/\sqrt{\delta}$ where a is the lattice parameter of the basal plane; for $\delta = 0.05$, $d \approx \xi_{ab}$, an almost ideal pinning situation. Unfortunately, oxygen deficiencies are not useful pinning centres, because their deleterious granular behaviour outweighs the benefits of the extra pinning that they provide.

Control of oxygen deficiency at the very low levels studied here is very difficult. As mentioned above, the single crystals are small and their oxygen content cannot be verified by thermogravimetric analysis (TGA). Equilibrium values of δ for 1 atm O_2 lie in the range 0.02–0.04 at 350–450 °C (ref. 17). After the last treatment given (480 h at 375 °C), the LPP crystal J_c values were virtually unchanged, whereas those for the flux-grown crystal were still decreasing. Even for polycrystals, where TGA is quite feasible, explicit checks of the degree of oxygenation are seldom made and measurement of the magnetization curve may provide the best guide to the oxygen content of the sample. Different defect densities (such as the difference between flux-grown and LPP crystals) must certainly be expected to affect the oxygenation kinetics.

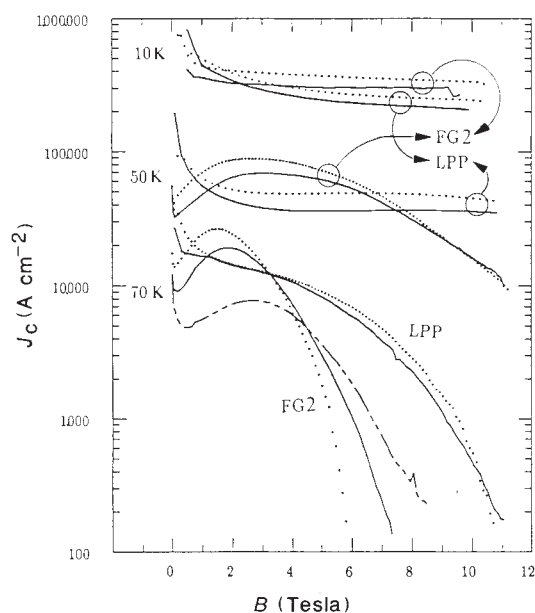


FIG. 4 Critical current density at various temperatures derived from the magnetization loops using the sample size as the scaling radius. Dotted lines correspond to low-temperature oxygenations of 300 h at 450 °C (LPP) and 200 h at 450 °C (FG2) and solid lines correspond to a further oxygenation of 200 h with slow cooling from 400 to 350 °C. The dashed curve corresponds to a further oxygenation of 480 h at 375 °C for the FG2 sample.

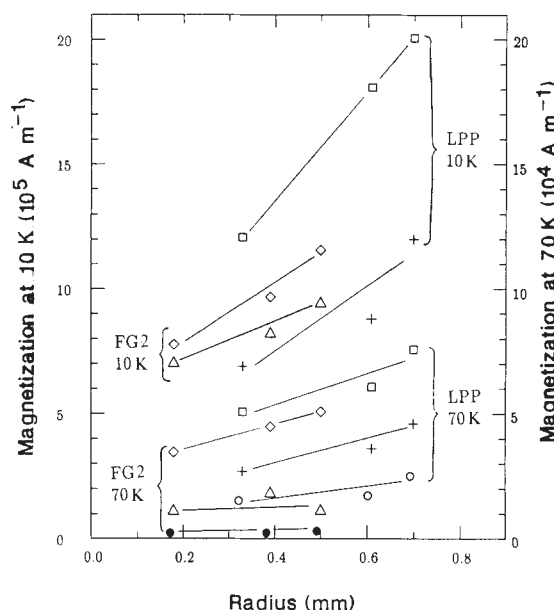


FIG. 5 Magnetization hysteresis measured at 10 K and 70 K at various fields for both the LPP and FG crystals. The radius was varied by subdividing the crystals. The oxygen condition was 200 h with slow cooling from 400 to 350 °C. LPP: $\square$, 1 T; $+$, 4 T; $\circ$, 6 T. FG2: $\diamond$, 1 T; $\triangle$, 4 T; $\bullet$, 6 T.

There are two final issues to discuss. The first concerns another aspect of the size-scaling results. In Fig. 5 it can be seen that, even in the regimes where $M \propto r$, none of the extrapolations go through the origin, as would be expected for fully coupled crystals. This suggests that there is some residual granularity for all conditions studied, consistent with several other studies²⁻⁴. It is noticeable, however, that the data for the LPP crystal, which show no anomaly, extrapolate to lower values of ΔM than is the case for the FG2 crystal, which does show an anomaly, and this is consistent with the through-origin size-scaling results on quench-melt-grown (QMG) crystals obtained by Murakami *et al.*⁶ in the range 0–1 T at 77 K. The second-phase and low-angle grain boundaries of LPP and QMG crystals should certainly enhance their oxygenation as compared with the more perfect FG crystals. Thus several granular properties that are sensitive to the precise defect structure of the crystal may exist⁴. The second point concerns the generality of our results. Although the nature of the samples is very different—two are second-phase-free single crystals grown by a flux route in different laboratories, whereas the third is a highly textured crystal containing some 20% of the 211 phase—all three samples exhibited qualitatively the same behaviour. Indeed, anomalous magnetization curves are very commonly observed in ‘good’ single crystals made in different ways^{4,11-13} and in polycrystalline samples^{4,21}. In fact, as discussed above, it is reasonable that samples generally have small oxygen deficiencies. At the present stage of high- T_c single-crystal studies, there are still many unknown material parameters and the effects reported here are clearly evident in precisely the types of crystals on which much of the basic physics of the compounds is being experimentally studied.

In summary, field-induced granular behaviour has been observed in three crystals of $YBa_2Cu_3O_{7-\delta}$. We have varied the oxygenation conditions of two crystals in such a way as to make their δ values small. Decreasing δ tends to eliminate the anomalous double-peaked magnetization curve generally found in single crystals of $YBa_2Cu_3O_{7-\delta}$ compounds. Further, decreasing δ decreases the flux pinning in the crystals and granular behaviour is seen above the second peak in the magnetization curve. We conclude that all three features, the anomalous magnetization curve, the flux pinning and the granular behaviour

are related to the oxygen deficiency, even when δ is of the order of 0.05. Explicit verification that these anomalies are absent is important for understanding the flux pinning, and the granular and reversible properties of the $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ compounds. In

particular we note that practical applications of these materials will require elimination of both the intra-grain weak links identified here, as well as the more generally recognized inter-granular weak links. □

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Movement of internalized ligand–receptor complexes along a continuous endosomal reticulum

C. R. Hopkins, A. Gibson, M. Shipman & K. Miller

Department of Biochemistry, Imperial College, London SW7 2AZ, UK

Complexes of cell-surface receptors and their ligands are commonly internalized by endocytosis and enter a prelysosomal endosomal pathway for further processing. Fluorescence microscopy and video recording of living cells to trace the passage of ligand–receptor complexes has identified the endosomal compartment as an extensive network of tubular cisternae. Endocytosed material entering this reticulum enters discrete swellings, identified as multivesicular bodies by electron microscopy, which move along the reticulum towards the pericentriolar area.

THE acidic endosomal compartment has a central role in the receptor-mediated uptake and processing of a wide range of metabolites (such as cholesterol) and pathogens, and in the recycling of receptor–ligand complexes^{1–3}. The membrane boundary of the endocytotic pathway is also probably involved in the transduction of mitogenic signals. Defining the limits of this system and identifying its regulatory mechanisms are therefore of considerable current interest in cell biology.

The endosome is a difficult organelle to isolate by conventional methods of subcellular fractionation because it lacks a characteristic buoyant density and is highly pleiomorphic. It has been studied most successfully by microscopical methods and some of its component parts have been described in detail^{4–6}. However, the overall form of the organelle and the way in which its diverse functions are regulated remain unclear.

We have applied various microscopical methods to define the form of the endocytotic pathway in living cells. Here we describe

confocal microscopy and low light video recording of living epidermoid carcinoma cells (Hep-2) to follow the movement of fluorescent tracers through the endosomal compartment. Contrary to expectation we have found an extensive tubular reticulum with expanded swellings along its length. Electron microscopy shows that these structures are typical multivesicular bodies, endosomal elements which have been identified previously in fixed cells and which hitherto had been thought to be free vacuoles.

Identification of an endosomal reticulum

The iron-carrying protein transferrin enters cells by receptor-mediated endocytosis. Transferrin labelled with the dye Texas

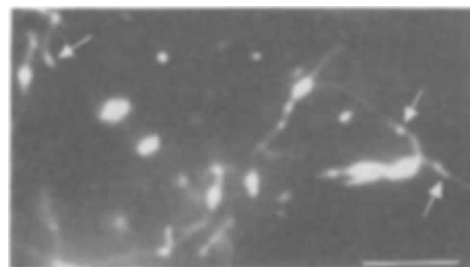


FIG. 1. Cisternal elements in peripheral cytoplasm of Hep-2 carcinoma cells. The fine branching cisternae have varicosities of varying sizes along them. Arrows indicate moving flows of tracer which on video show direction and rate of movement. Cells were grown in Dulbecco's MEM plus 5% fetal calf serum in sparse culture as described previously for A431 cells⁷. They were incubated in subsaturating (10^{-9} M) concentrations of TF-TXR for 60 min or more at 37 °C. Image obtained with a Hamamatsu C 2400-08 SIT camera. Scale bar, 5 μm .

red (TF-TXR) was used as tracer for the endosomal pathway in living cells. To localize internalized receptors, anti-transferrin receptor antibodies (ATR) tagged either with the fluorescent label FITC or (for electron microscopy) with gold, were used as probes.

The endosomal system was loaded to steady state⁷ by incubating Hep-2 cells for 60 min or more at 37 °C with subsaturating concentrations of TF-TXR which binds to transferrin receptors. Confocal and low light video recording with an SIT camera then identified and followed the tracer as it moved through the cells. Confocal microscopy was used to compare the distribution of fluorescent- or gold-labelled anti-receptor antibodies.

Previous studies on fixed cells have shown internalized transferrin tracers to be distributed throughout the cell within irregularly shaped but often elongated elements^{7,8}. Using the SIT camera to follow the movement of the fluorescent tracers within living cells it was clear that many of these elements were interconnected by fine tubular cisternae (Fig. 1). Even though the system was loaded to steady state the tracer was often seen to move as a concentrated bolus through these tubules. As a rule the moving bolus did not alter the dimension of the tubules and by measuring the displacement of these concentrations with time, rates of movement in the range 0.5 to 1.0 $\mu\text{m s}^{-1}$ were obtained. The tubules often branched to form interconnected networks (Fig. 2) and within these networks concentrated flows

of tracer were seen to break into two separate streams as they moved through a branch point. This pattern of behaviour was displayed by both TF-TXR and ATR-FITC tracers and as pulse-chase experiments using radiolabelled TF-TXR show the same kinetics of uptake and recycling as [¹²⁵I]transferrin (data not shown) we believe that the system outlined is endosomal and separate from the tubular lysosomal elements described in previous reports^{9,10}.

Brightly fluorescent swellings or 'varicosities' with diameters of between 0.1 and 0.6 μm occurred along the tubular cisternae at irregular intervals (Fig. 1). These varicosities also moved along the tubules, a movement which could be readily distinguished from the moving concentrations of tracer described above because the localized dilations themselves were displaced. Also, as described in detail below, varicosities move much more slowly.

The tubular reticulum could be found in all parts of the cell but did not seem to form a single, continuous organelle. The longer cisternae (<5 μm) were frequently orientated towards the centrioles and were sometimes seen to extend from the peripheral cytoplasm, just below the plasma membrane, down into the pericentriolar area. Tracer moving within these tubules did not seem to follow any preferred direction (such as towards the nucleus).

The finer tubules in the reticulum were very susceptible to breakdown. Ultraviolet illumination for more than a few minutes, low temperature (20 °C), increased pH or other suboptimal culture conditions caused it to break up into short tubulovesicular elements. These fragmented elements usually exhibited a hesitant, saltatory movement¹¹.

EM of transferrin-receptor complexes

The fate of transferrin-receptor complexes was also followed by electron microscopy. Cells were labelled with gold-anti-receptor antibody. In plastic sections, as described previously^{7,12}, gold-labelled elements in the peripheral cytoplasm appeared as tubulo-vesicles 30–50 nm in diameter and vacuoles 0.3–0.5 μm in diameter. Only in thick 1- μm sections was there any indication of a cisternal network.

Many of the 0.3–0.5 μm vacuoles lying immediately below the plasma membrane (Fig. 3a, b) displayed a distinctive cup-shaped form. The origin of these structures, which after short incubations with tracer were the first to be labelled, was not apparent. In vacuoles which become labelled after the cup-shaped structures there were usually two or three internal vesicles within the lumen. Most of the vesicles within these multivesicular bodies (MVBs) were 30–50 nm in diameter and at this time they were closely associated with the perimeter membrane (Fig. 3d). Frequently, there was also a larger (~0.3 μm diameter), centrally placed vacuole.

The ATR-gold label was associated exclusively with the inner face of the perimeter membrane (Fig. 3c–e), and does not enter the internal vesicles.

Processing of EGF-receptor complexes

The endocytotic pathway transfers some ligand-receptor complexes to the lysosome while recycling others to the plasma membrane and so must contain a mechanism for sorting receptor populations. To shed light on this problem we have labelled epidermal growth factor (EGF)-receptor complexes¹³ and followed their fate in the endosomal pathway. Internalization of EGF receptors is induced by the binding of EGF. To correlate light and electron microscopical preparations directly we have used an antibody (108) which binds to EGF receptors without inhibiting EGF binding or inducing internalization^{14,15}. By coupling this 108 antibody to fluorescent (FITC) or gold labels the trafficking of EGF-receptor complexes could be followed by both light and electron microscopy. In double-label experiments (Fig. 4) we were able to show that fluorescent and gold labels colocalized.

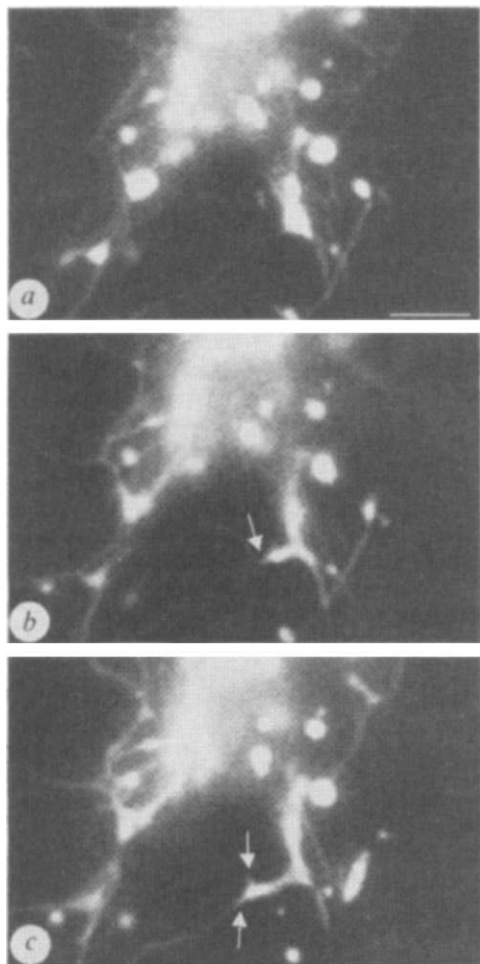


FIG. 2 Endosomal elements in the pericentriolar area loaded with TF-TXR show a complex of bulbous varicosities interconnected by fine branching tubular cisternae. *b* and *c* taken from video 40 s apart. Arrows indicate flow of tracer moving into bifurcating branches of cisterna. Preparation and video recording using SIT camera as in Fig. 1. Scale bar, 5 μm .

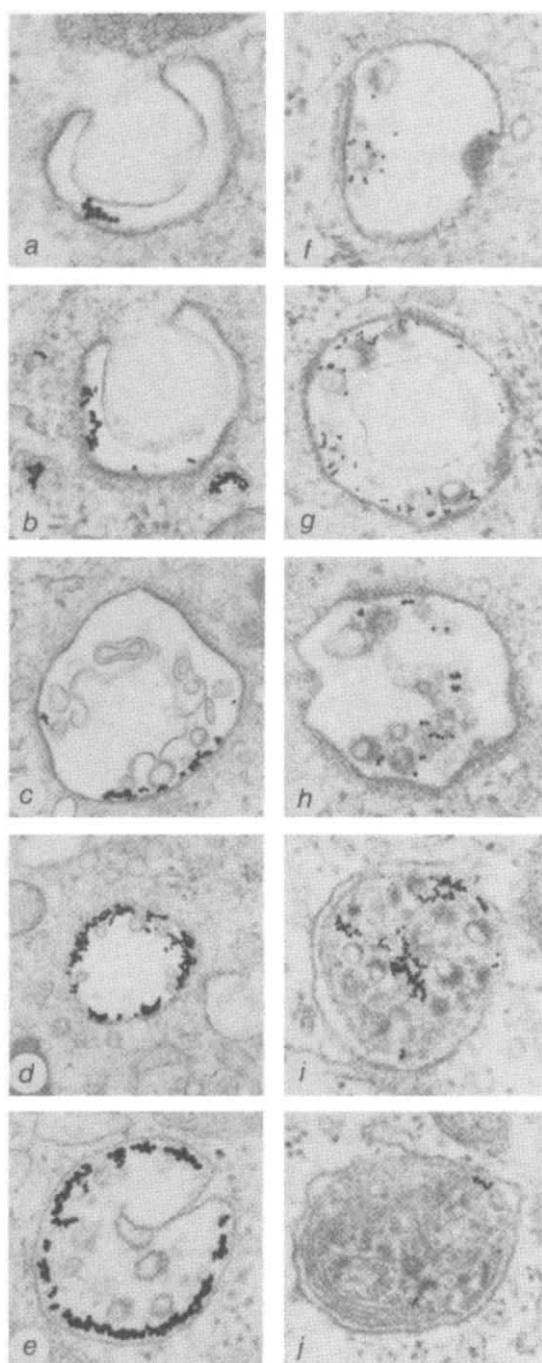


FIG. 3 Electron micrographs showing endosomal elements varying in size between 0.3 μm and 2.0 μm in diameter, containing ATR-gold (*a–e*) or gold-labelled anti-EGF receptor antibody (*f–j*). *a, b*, Earliest labelled cup-shaped vacuoles; *c*, multivesicular body with label on its perimeter membrane; *d, e*, multivesicular bodies from the pericentriolar area containing internal vesicles, the gold label remaining on the perimeter membrane; *f*, earliest stage of 0.3–0.5 μm diameter vacuole found. EGF receptor label is on an internal vesicle and there is a local invagination of the perimeter membrane on the right hand side of the vacuole; *g*, multi-vesicular body similar to *b* but cut equatorial to the cup-shaped invagination and showing EGF receptor label on small internal vesicles; *h, i*, multivesicular bodies with increasing numbers of EGF receptor-labelled internal vesicles; *j*, labelled lysosome-like structure from the pericentriolar area. Cells were prepared as in Fig. 1 and incubated with gold-antibody conjugates at 5 $^{\circ}\text{C}$ for 30 min, warmed and incubated with 20 nM EGF for 3–30 min at 37 $^{\circ}\text{C}$ before being fixed and prepared for electron microscopy. All reagents and procedures as described previously⁷. Scale bars, 100 nm.

of a U-shaped tubule and tracer is filling the tubule to its rear. When the varicosity reaches a branchpoint in the reticulum the transferrin tracer can by-pass the restriction caused by the varicosity and flow rapidly away into ill defined side branches (Fig. 5c). As this occurs, the length of the transferrin tracer in the tubule behind the varicosity is reduced.

In the electron microscope the distribution of 108-gold was the same as that shown previously for internalized EGF-receptor using cryosection immunocytochemistry⁶. These results suggest therefore, that receptor sorting occurs in the MVBs/varicosities, as EGF-receptor complexes, but not transferrin-receptor complexes, enter the internal vesicles of the MVBs and are then transported towards the pericentriolar area. At the earliest time points after EGF stimulation 108-gold complexes were present on the perimeter membranes of 0.3–0.5 μm vacuoles but were not seen in the cup-shaped elements. From 3 min onwards they were found increasingly on the internal vesicles of MVBs (Figs 3g, h, i). From 20 min after EGF stimulation MVBs packed with 108-gold labelled internal vesicles began to accumulate in the pericentriolar area (Fig. 3i). From 45 min onwards the gold-labelled vacuoles contained fewer vesicles but included a variety of membrane whorls and myelin figures typical of lysosomes (Fig. 3j).

Transport along the endocytotic pathway

Our studies show that the endocytotic pathway includes a branching reticulum with tubular elements up to several micrometres long. The discovery that the endocytotic pathway is more continuous than is apparent in fixed preparations does not alter the consensus view of the organelle as a membrane-bound

Initially, EGF caused 108-FITC to internalize into elements 0.1–0.5 μm in diameter distributed throughout the peripheral cytoplasm, but with time (≤ 30 min) the label concentrated in the pericentriolar area. In cells incubated to steady state with TF-TXR the EGF-receptor could be detected within the varicosities of the transferrin-labelled cisternae. In EGF-stimulated cells the tubules outlined by TF-TXR were greatly elongated and there seemed to be a significant increase in the number of varicosities throughout the cell. Close examination with the SIT camera showed that many of the varicosities were, themselves, moving along the tubules. They moved at a rate of approximately 0.05 $\mu\text{m s}^{-1}$ and seemed to retard the flow of the faster moving concentrations of tracer (described above) because the tracer could often be seen backed up in the tubules behind them. An example of varicosity movement is shown in Fig. 5. In this sequence a varicosity is moving down one side

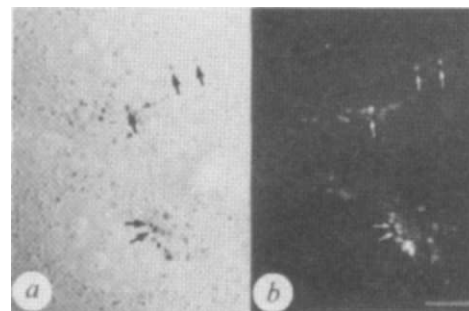


FIG. 4 Colocalization of (*a*) gold-labelled EGF receptor (with antibody 108-gold) and (*b*) fluorescently labelled EGF receptor (with antibody 108-FITC). Arrows indicate the same discrete compartments labelled in both fields. Cells incubated with 108-FITC and 108-gold for 60 min at 4 $^{\circ}\text{C}$, then stimulated with EGF. Scale bar, 10 μm .

processing compartment of low pH. It does suggest, however, that many of the molecular interactions that underlie the selective trafficking of membrane proteins and signal transduction should now be looked for within the planes of single membrane boundaries. Separate vesicular compartments and selective vesicle fusions may not be as important as recent studies on cell-free systems suggest¹⁶. Moreover, alternative ways of generating microenvironments of lowered pH may now need to be considered.

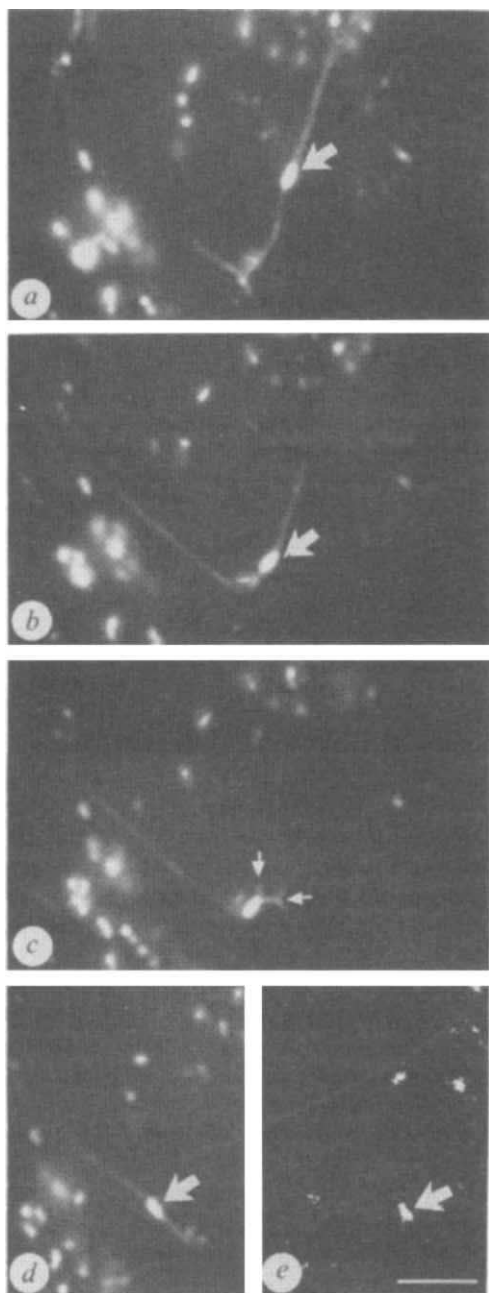


FIG. 5 Branching tubular cisterna showing multivesicular body/varicosity moving along it. Video frames taken from a sequence of images 30 s apart. In *a–b* the varicosity (arrowed) is moving down the right arm of the cisterna. At *c–d* it moves around the bend and up the left arm. Small arrows in *c* indicate side branches into which the tracer TF-TXR flows once the varicosity moves beyond them. *e*, FITC image showing distribution of EGF receptor (labelled with 108-FITC). Arrow indicates the same varicosity as in *d*. Cells cultured as in Fig. 1 then incubated at 5 °C for 30 min with 108-FITC and 20 nM EGF, then rinsed and incubated for 30 min with TF-TXR at 37 °C. Scale bar, 5 μ m.

Processing of ligand–receptor complexes

Our finding that the varicosities of the endosomal reticulum seen in living epithelial cells are MVBs shows that the lumina of these vacuolar structures are confluent with the cisternae of the reticulum. Our observations further suggest that trafficking proteins are carried to the MVBs through the tubules. Many previous studies have shown that MVBs with tubulo-vesicular extensions contain ligand–receptor complexes and fluid-phase tracers^{10,17}. There is also good immunocytochemical evidence to show that vacuolar structures with tubular arms (CURL) are an important site of ligand–receptor dissociation⁴. Detailed models of the way in which receptors might be removed from MVBs into their cisternal extensions have been proposed¹⁸ but the possibility that ligand–receptor complexes are being delivered to the MVB by way of a reticulum has not been considered. From our observations it now seems likely that ligand–receptor complexes flow through the MVBs and that as they do they are subjected to dissociating conditions such as transiently lowered pH. In a recent quantitative study Dunn *et al.*¹⁹ showed that as more fluorescent tracer enters the endocytotic pathway, vacuolar endocytotic elements in the same size range as MVB become brighter rather than more numerous. It is possible, therefore, that several passages may be required before MVB processing (which in addition to dissociation might include conformational change, breakage of disulphide bonds and proteolytic cleavage)^{3,20} is complete.

MVB movement and removal of membrane proteins

The view of the endosome system as consisting of an 'early' compartment processing rapidly recycling receptors and a 'late' compartment reached after longer intervals (<10 min) is compatible with the observations made in this study. It is now possible, however, to put this view in the context of a reticulum (through which ligand–receptor complexes moved at a rapid rate) and MVB/varicosities (into which some of these complexes become sequestered) in which they are then carried, more slowly, into the pericentriolar area. The rate at which the MVB are seen to move could depend upon a microtubular mechanism as suggested in previous studies^{21,22}.

Because EGF receptors are found initially on the perimeter membrane of the endosomal reticulum we conclude, as in our earlier studies^{6,15}, that the EGF-rich internal vesicles of the MVB arise by the invagination of receptor-rich domains on this boundary membrane. Movement of the MVB through the reticulum explains how such a large number of internal vesicles could be produced. The amount of membrane required to form these vesicles is far in excess of that available in the perimeter membrane of an isolated vacuole. In a moving varicosity the perimeter membrane will bring in a continuous supply of membrane. This process of 'microautophagy' suggests a system in which varicosities moving through the system gradually accumulate internal vesicles so that by the time they reach the pericentriolar area they will be loaded with internal membranes.

These studies, along with other recent reports of previously undetected cisternal compartments within exocytotic and endocytotic routes^{9,23,24}, suggest much greater continuity within intracellular pathways than did previous work which relied on chemically fixed preparations. They help to explain some apparent functional anomalies, such as the asynchrony of membrane trafficking which is frequently observed along these pathways, and they suggest that local sequestration within a cisternal compartment (rather than within separate vesicles) is likely to be a much more important feature of intracellular processing than has previously been realized. □

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LETTERS TO NATURE

DNH deoxyribonucleohelicates: self assembly of oligonucleosidic double-helical metal complexes

Ulrich Koert, Margaret M. Harding* & Jean-Marie Lehn†

Laboratoire de Chimie Supramoléculaire, Institut le Bel, Université Louis Pasteur, 4, rue Blaise Pascal, 67000 Strasbourg, France

NUCLEIC acids, because of their key biological role, are prime targets for the design of either analogues that may mimic some of their features or of complementary ligands that may selectively bind to and react with them for regulation or reaction. Whereas there has been much work on the latter topic since the elucidation of the double-helical structure of DNA^{1,2}, comparatively little has been done on structural and/or functional models, probably owing to the lack of self-organizing molecular systems. Here we present a class of artificial systems, the nucleohelicates, which are of interest from both points of view because they combine the double-helical structure of the double-stranded metal complexes, the helicates^{2,3}, with the selective interaction features of nucleic-acid bases. These functionalized species allow the study of structural effects on the formation of the double helix and on the binding to other entities, in particular to nucleic acids.

We have shown previously that oligobipyridine molecules containing a string of 2,2'-bipyridine (bipy) groups separated by a suitable spacer unit undergo spontaneous self-organization into oligonuclear metal complexes with a double-helical structure (Fig. 1). In these helicates two molecular strands are wrapped around each other and held together by Cu(I) ions that provide the driving force for organization by imposing a (distorted) tetrahedral coordination geometry at each site. Di- to pentahelicates have thus been obtained^{2,3}. The trihelicate [Cu₃(3-H)]³⁺, (4-H), schematically represented by structure **3**, is formed by the binding of three Cu(I) ions by two tris-bipyridine molecules (3-H); it has been characterized by crystal structure determination². These compounds provide a molecular framework for the spatial organization of substituent groups attached to the periphery of the double helix and projecting outwards. Exploitation of this possibility requires the introduction of functional groups on the bipyridine subunits that will allow the attachment of various molecular groups.

Two types of functionalized oligobipyridine compounds have been synthesized: fully substituted ones such as 3-X₆ and partially substituted ones, 3-X₄ and 5-X₆, where substituted and unsubstituted bipyridine units alternate along the strand (Fig. 2). They were obtained following a scheme similar to that used for the synthesis of the unsubstituted parent compounds 3-H (ref. 2) and 5-H (ref. 3) from the appropriate basic units (X, X' = H, OH or Br; Y = CO₂t-Bu or CH₂CH₂CO₂t-Bu) derived from

the diester **6** (X = X' = H, Y = CO₂Me)⁴ using reactions to be described elsewhere.

Compounds 3-E₆, 3-(C₂E)₆, 3-(C₂E)₄ and 5-(C₂E)₆ allow the introduction of a variety of groups (such as nucleosides, aminoacids, sugars, electroactive and photoactive units) on the oligobipyridine strands. Only one case, that of an aminodeoxy-nucleoside, 6'-amino-3'-deoxythymidine (H-Thy), used as its soluble silylated derivative (H-ThyS), is described here.

The corresponding derivatives 3-T₆, 3-(C₂T)₆, 3-(C₂T)₄ and 5-(C₂T)₆ represent artificial oligonucleosidic strands in which four or six nucleoside residues are attached to a backbone of bipyridine units. The analogous aminodeoxy-adenosine derivative 5-(C₂A)₆ has also been synthesized. Such molecules may, for instance, allow the study of base-pairing features in model arrays of nucleic acid components and the design of novel systems presenting recognition and self-assembling properties. They also represent alternatives to flexible oligonucleoside analogues⁵. Pendant nucleic-acid bases have been introduced on the backbone of organic polymers^{6,7} and a number of systems containing two nucleic bases have been reported (see, for example, refs 8–14).

Complexation of Cu(I) by the unsubstituted oligobipyridine compounds 3-H and 5-H has been shown to give, respectively, the tri- and pentahelicates^{2,3}. Similarly, the substituted derivatives described here gave the corresponding substituted helicates when the polyesters 3-E₆, 3-(C₂E)₄, 3-(C₂E)₆ and 5-(C₂E)₆ were treated with copper(II) trifluoro CuTf₂ (Tf, CF₃SO₃) (2 eq) and hydrazine (N₂H₄·H₂O) in CH₃CN or in CH₃CN–CHCl₃; these results will be described elsewhere. The 200-MHz ¹H-NMR and FAB⁺ (fast-atom bombardment) mass spectra of the octaester and dodecaester helicates {Cu₃[3-(C₂E)₄]₂}Tf₃, 4-(C₂E)₈ and {Cu₅[5-(C₂E)₆]₂}Tf₅ are shown in Figs 3a, c, 4a and 5a; they agree with the structures, as do the elemental analyses.

A deep-red colour characteristic of Cu(I)-bipyridine complexes was also obtained on treatment of 3-T₆ with the same reagents in 1:1 aqueous acetonitrile. The NMR and mass spectral data did not confirm the formation of only the dodecathymidino-trihelicate, but indicated that a mixture of complexes was obtained. This may be due to the destabilizing effect of both the carbonyl groups conjugated with the pyridine rings and steric hindrance between the bulky substituent groups. Accordingly, we introduced a CH₂CH₂ segment into the side chains on alternating bipyridine units, as in 3-(C₂T)₄ and 5-(C₂T)₆, to reduce both adverse effects.

The following typical procedure was followed for preparing the complexes. 30.0 mg (1.35 × 10⁻⁵ mol) of 3-(C₂T)₄ were dissolved in 15 ml CHCl₃ at room temperature and a solution of 14.7 mg (4.06 × 10⁻⁵ mol, 3 eq) of copper(II) trifluoro CuTf₂ in 5 ml CH₃CN added. The blue-green solution of the Cu(II) complex thus obtained was stirred for 10 min. Then, addition of 30 μl (6.18 × 10⁻⁴ mol, 15 eq) of N₂H₄·H₂O reduced Cu(II) to Cu(I) and gave the expected colour change to red. The reaction mixture was stirred for 15 min. After evaporation of the solvent, the

residue was taken up in 20 ml CHCl_3 and filtered through cotton wool. By addition of 30 ml of ether, the product was isolated as a red precipitate (29.9 mg, 5.89×10^{-6} mol, 87%; m.p. 180–181 °C). Following the same procedure, treatment of 26.0 mg of $\mathbf{5}-(\text{C}_2\text{T})_6$ with 5 eq of CuTf_2 and 20 eq of $\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$ in $\text{CH}_3\text{CN}-\text{CHCl}_3$ gave 25.1 mg of a red-orange solid (m.p. 178 °C; 88% yield).

The elemental analyses (C, H, N, Cu) and ultraviolet absorption data of these solids were in agreement with the compositions $[\mathbf{3}-(\text{C}_2\text{T})_4]_2[\text{CuTf}]_3$ and $[\mathbf{5}-(\text{C}_2\text{T})_6]_2[\text{CuTf}]_5$.

The FAB⁺ mass spectra, with peaks corresponding to $\{[\mathbf{3}-(\text{C}_2\text{T})_4]_2\text{Cu}_3\text{Tf}_2\}^+$ (Fig. 3b) and $\{[\mathbf{5}-(\text{C}_2\text{T})_6]_2\text{Cu}_5\text{Tf}_4\}^+$ (Fig. 3d), proved the presence of two ligands and, respectively, three and five copper(I) ions.

The ^1H -NMR spectra of the polynucleosidic trihelicate and pentahelicate were consistent with the assigned structures. High-field two-dimensional NMR techniques were used to analyse the spectra. In principle, complexation should lead to a mixture of the two diastereomers that differ in the handedness of the double helix. The presence of the chiral nucleosides might lead to a preferential induction of one helical sense, but this has not yet been proven. Figure 4b (left) shows the NMR and TOCSY (total correlation spectroscopy) spectra of $\{\text{Cu}_3[\mathbf{3}-(\text{C}_2\text{T})_4]_2\} \text{Tf}_3$, compound **1**. Changes in the ligand chemical shifts upon copper complexation were consistent with those observed earlier² on formation of the unsubstituted trihelicate **4-H** from **3-H**. The first change is a marked upfield shift and splitting of the

CH_2OCH_2 singlets into two multiplets (O) corresponding to two AB systems in **4-H**. The cross-peak indicated in the TOCSY spectrum (O) results from the coupling in the two AB systems. The second change is an upfield shift of the two bipyridine protons (3a, 3b) and of the bipyridine methyl group (Me); it may be attributed to shielding by pyridine units of the other strand. The interpretation was confirmed by comparison with the ^1H -NMR spectrum of the octaester helicate **4-(C₂E)₈** (Fig. 4a, left), which shows similar chemical shifts and for which the same changes are observed on helicate formation.

In an analogous manner the ^1H -NMR spectrum (NMR and TOCSY) of $[\mathbf{5}-(\text{C}_2\text{T})_6]_2[\text{CuTf}]_5$ (Fig. 4b, right) is consistent with a dodecathymidino-pentahelicate $\{\text{Cu}_5[\mathbf{5}-(\text{C}_2\text{T})_6]_2\} \text{Tf}_5$, **2**. The most important spectral features are again the AB multiplets (O) (cross-peak O in the TOCSY spectrum). Comparison with the spectrum of the corresponding dodecaester helicate $\{\text{Cu}_5[\mathbf{5}-(\text{C}_2\text{E})_6]_2\} \text{Tf}_5$ (Fig. 4a, right) again confirms the structural and spectral assignments. Water-soluble derivatives of compounds **1** and **2** (R = H, sulphate salts) have been obtained from the corresponding desilylated strands.

In view of its features this new class of substances may be termed deoxyribo-nucleohelicate, DNH. The DNH complexes, such as compounds **1** and **2**, are double-helical oligonucleosides formed spontaneously by self-assembling of two suitably designed ligand strands and copper(I) ions. In contrast to DNA they have positive charges located inside the strands whereas the information-bearing nucleic-acid bases are on the outer spine

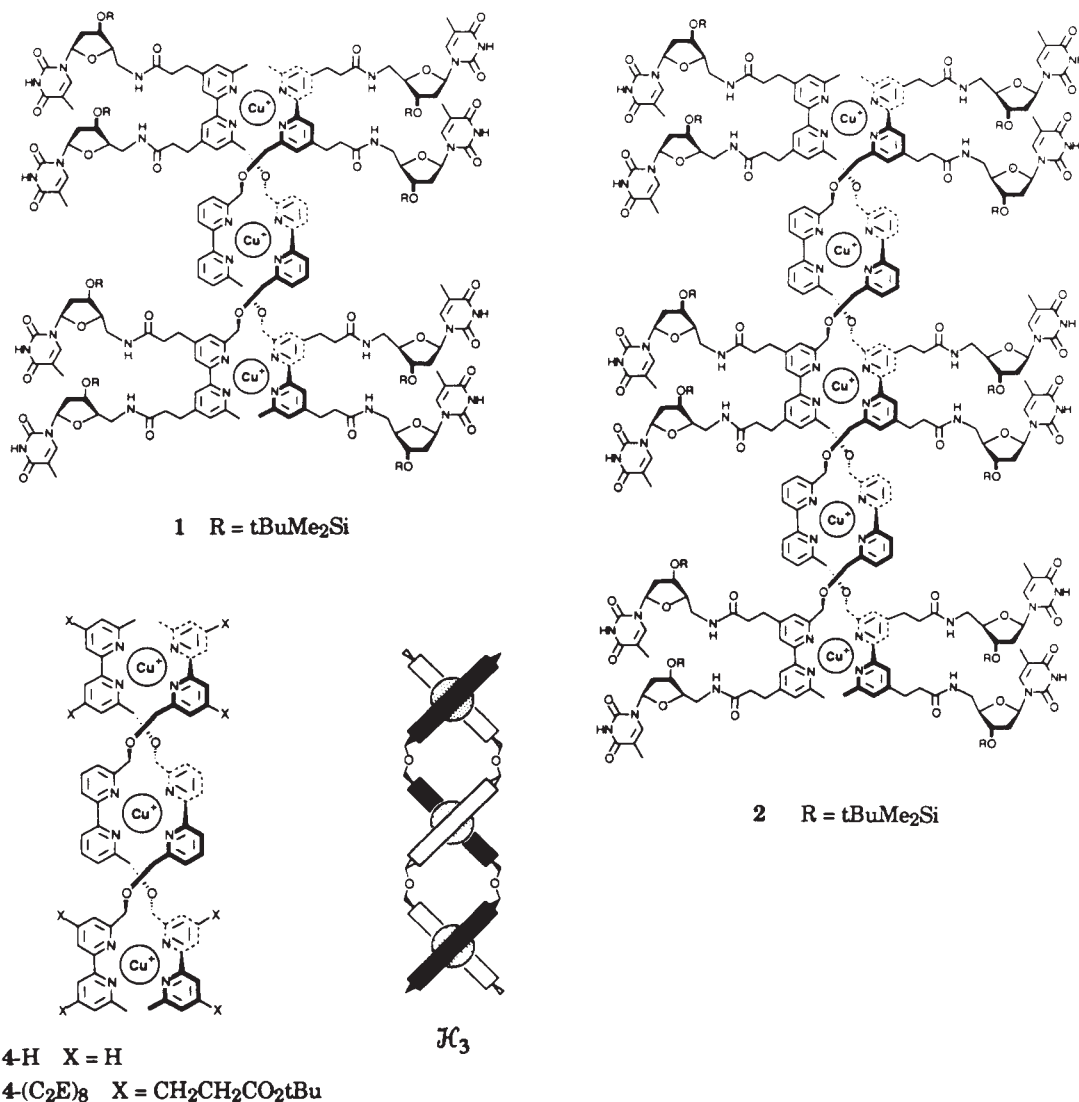
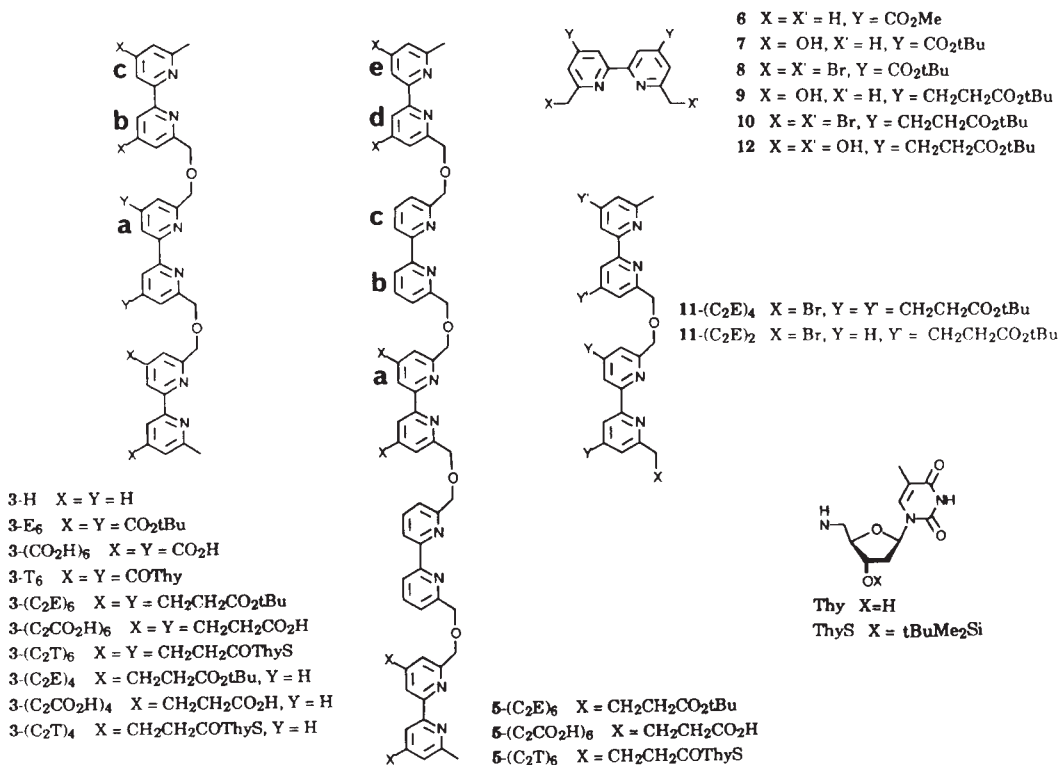


FIG. 1 Structure of the deoxyribonucleohelicates **1** and **2** and of the trihelicates **4-H** and **4-(C₂E)₈**; the two bipyridine groups at each Cu(I) centre are almost perpendicular to one another, as schematically represented in $\mathcal{H}_3$ for **4-H**.

FIG. 2 Oligonucleosidic strands **3-T**₆, **3-(C₂T)**₆, **3-(C₂T)**₄ and **5-(C₂T)**₆ and synthetic intermediates.

METHOD. The key reaction was the condensation of compounds **7** and **8**. *t*-BuOK (2.4 eq) was added to a solution of compound **8** (2 eq) in *t*-BuOH and stirred at room temperature. After 1 h a solution of compound **7** (1 eq) in CH₂Cl₂ was added and the mixture was refluxed for 4 h. Together with the presence of the *t*-Bu groups in the ester functions, a bulky base and solvent were used to minimize side-reactions. The trimeric hexaester **3-E**₆ was isolated and purified by chromatography on silica (m.p. 135–136 °C, 50% yield). Condensation of 2 eq of the monoalcohol **9** with the dibromide **10** using *t*-BuOK (1:1 *t*-BuOH:THF, 20 °C, 24 h) gave elongated hexaester **3-(C₂E)**₆ (m.p. 76 °C, 31% yield) together with the bis-bipy monobromide **11-(C₂E)**₄ (oil, 23% yield), which were separated by column chromatography (alumina; eluent, 1:4 AcOEt:hexane). Condensation of 2 eq of the monoalcohol **9** with 6,6'-bis-bromomethyl bipy²⁰ using *t*-BuOK (1:1 *t*-BuOH:THF, 20 °C, 4 h) gave a mixture of the mono-substituted dimeric species **11-(C₂E)**₂ (m.p. 88 °C, 57% yield) and the alternating tris-bipy tetraester **3-(C₂E)**₄ (m.p. 48 °C, 30% yield), which were separated by column chromatography (alumina; eluent, 4:1 hexane:AcOEt). Condensation of 2 eq of the bis-bipy monobromide **11-(C₂E)**₂ with the dialcohol **12** (*t*-BuOK; 1:1 *t*-BuOH:CH₂Cl₂, room temperature 12 h) gave the alternating penta-bipy elongated hexaester **5-(C₂E)**₆ which was isolated and purified by chromatography (alumina; eluent, 2:1 hexane:AcOEt, m.p. 108 °C, 47% yield). 6'-Amino-3'-deoxy-thymidine (H-Thy) and its 3'-*t*-butyldimethyl silyl ether (H-ThyS) were prepared from thymidine following a reported reaction sequence²¹. The polyacids **3-(CO₂H)**₆, **3-(C₂CO₂H)**₆, **3-(C₂CO₂H)**₄ and **5-(C₂CO₂H)**₆ were obtained by acid-catalysed cleavage of the corresponding *t*-butyl esters (CF₃CO₂H, CH₂Cl₂, room tem-



perature 12 h; >95% yield) and transformed into the corresponding acid chlorides (SOCl₂, reflux, 1–2 h), which were used directly for condensation with either H-Thy or H-ThyS (2 eq per acid chloride function, 4 eq Et₃N, CHCl₃, 20 °C, 2 h) to give the corresponding oligonucleosidic compounds **3-T**₆ (43% yield), **3-(C₂T)**₆ (143 °C, 48% yield), **3-(C₂T)**₄ (167–168 °C, 70% yield) and **5-(C₂T)**₆ (162–163 °C, 54% yield). The silylated compounds **3-(C₂T)**₆, **3-(C₂T)**₄ and **5-(C₂T)**₆, which were readily soluble in polar organic solvents (such as methanol, chloroform) were purified by column chromatography (silica, THF eluent). On the other hand **3-T**₆ had very low solubility in most common solvents except dimethyl sulphoxide (DMSO); its solubility could be increased by acylation of the free hydroxyl function to the hexa-acetyl-, -butyryl-, -methoxyacetyl- and -decanoyl derivatives. All new compounds had spectral (NMR, FAB⁺ mass) and elemental analysis data in agreement with their structure.

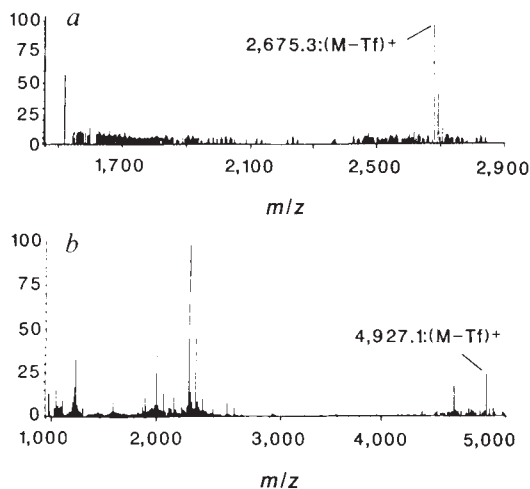
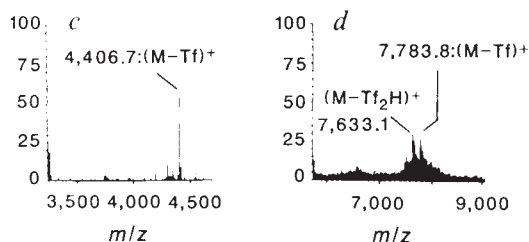


FIG. 3 Partial FAB⁺ mass spectra of **a**, Cu₃[**3-(C₂E)**₄]₂Tf₃: (M-Tf)⁺ found at 2,675.4 (calc. 2,675.3) (NBA, o-nitrobenzylalcohol); **b**, Cu₃[**3-(C₂T)**₄]₂Tf₃: (M-Tf)⁺ found at 4,927.1 (calc. 4,926.5) (DMSO); **c**, Cu₃[**5-(C₂E)**₆]₂Tf₅: (M-Tf)⁺ found at 4,406.7 (calc. 4,406.3) (NBA); **d**, Cu₃[**5-(C₂T)**₆]₂Tf₅: (M-Tf)⁺ found at 7,783.8 (calc. 7,782.8) (DMSO).



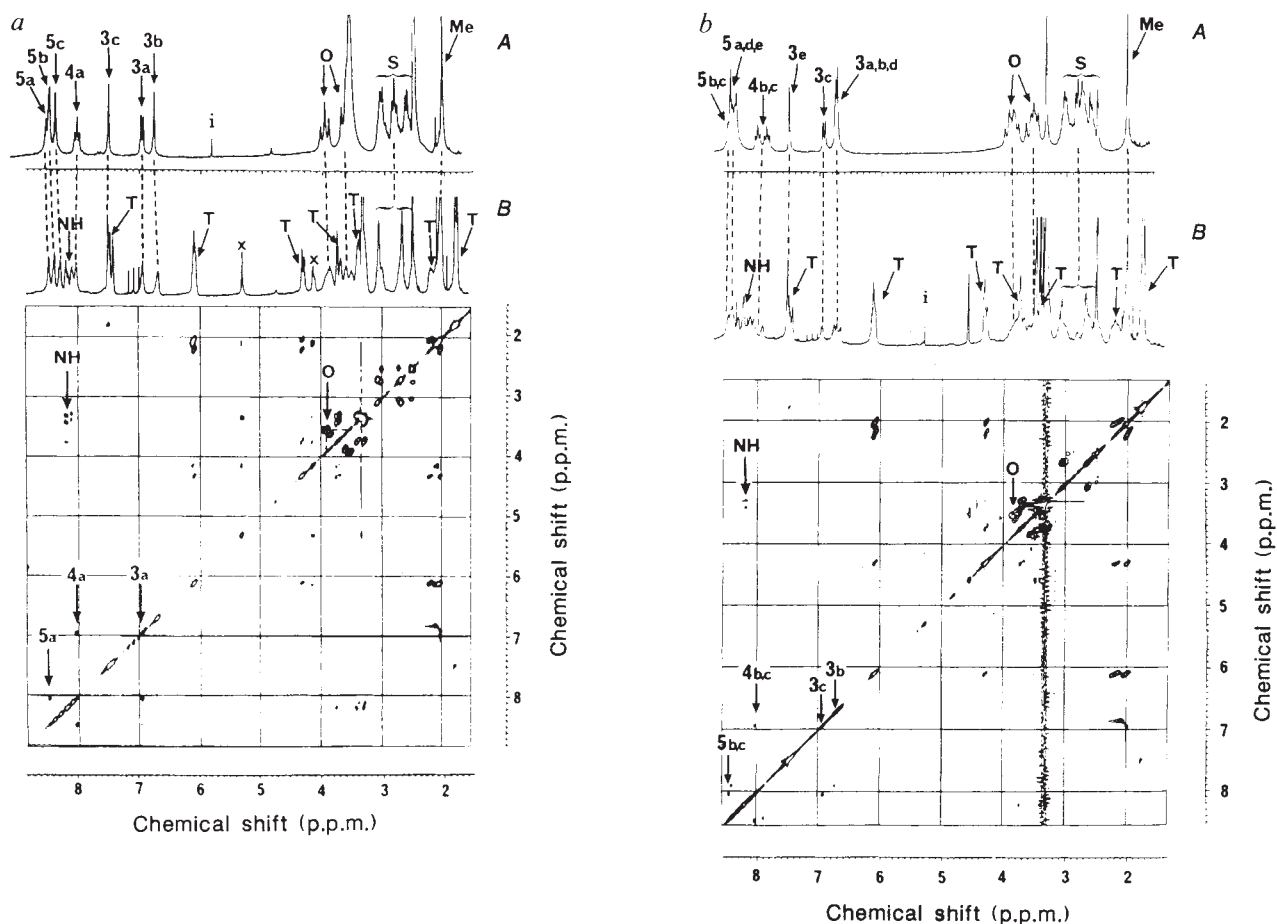


FIG. 4 ^1H -NMR spectra (200 MHz, 293 K) between 1.5 and 8.5 p.p.m. of the octaester trihelicate ($\text{Cu}_3(\mathbf{3}\text{-C}_2\text{E}_4)_2$) Tf_3 in d_6 -DMSO (aA) and the dodecaester pentahelicate ($\text{Cu}_5(\mathbf{5}\text{-C}_2\text{E}_6)_2$) Tf_5 in d_6 -DMSO (bA) showing the aromatic region (3, 4, 5), the methylene oxy bridge (O), the two-carbon spacer(S) and the bipyrindine methyl group (Me) signals. ^1H -NMR (600 MHz, 293 K) and TOCSY spectra of the octathymidine trihelicate **1** in d_6 -DMSO (aB) and the dodecathymidine pentahelicate **2** in d_6 -DMSO (bB). Helicate signals having

the same chemical shift as in aA and bA are indicated by dashed lines, thymidine signals by T, and propionamide NH by NH. Cross-peaks are marked by arrows for protons 3, 4, 5 on the bipyrindine ring systems a-e, the propionamide NH and the methylene oxy bridge AB systems. x, thymidine monomer impurity; triplet at 7.1 p.p.m., NH_4^+ ; solvent peaks at 2.5 (DMSO) and 3.5 or 3.3 p.p.m. (H_2O); i, solvent impurity; a-e refer to the different pyridine rings (see Fig. 2).

of the double helix, pointing away from its axis; this structure recalls the inside-out model once proposed for DNA¹⁵. They should be able to interact with other species both electrostatically and by hydrogen bonding through the thymidines (in a metallo-nucleate exoreceptor¹⁶ fashion). One possible target is the negatively charged double-stranded DNA; binding in its major

groove would lead to a composite natural-artificial quadruple-helix entity which might present thymine·adenine·thymine interactions (of the triple helix type¹⁷⁻¹⁹) with adenine-thymine regions in the DNA. Such possibilities are being explored. □

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Prediction of crystal structures from crystal chemistry rules by simulated annealing

J. Pannetier*, J. Bassas-Alsina†, J. Rodriguez-Carvajal* & V. Caignaert‡

* Institut Laue-Langevin, 156X, 38042 Grenoble Cédex, France

† Instituto de Ciencia de Materiales de Barcelona, Martí i Franqués, Barcelona 08028, Spain

‡ Laboratoire de Cristallographie et Sciences des Matériaux, Institut des Sciences de la Matière et du Rayonnement, 14032 Caen Cédex, France

THE prediction of the structure of inorganic crystalline solids from the knowledge of their chemical composition is still a largely unresolved problem^{1–3}. The usual approach to this problem is to minimize, for a selection of candidate models, the potential energy of the system with respect to the structural parameters of these models: the solution is the arrangement that comes out lowest in energy. Methods using this procedure may differ in the origin (*ab initio* or empirical) of the interatomic potentials used, but they usually restrict themselves to optimizing a structural arrangement within the constraints of given symmetry and bond topology. As a result, they do not truly address the problem of predicting the unknown structure of a real compound. The method we describe here is an attempt at solving the following problem: given the chemical composition of a crystalline compound and the values of its unit-cell parameters, find its structure (topology and bond distances) by optimizing the arrangement of ions, atoms or molecules in accordance with a set of prescribed rules. The procedure uses simple, empirical crystal chemistry arguments (Pauling's principles for ionic compounds⁴) and a powerful stochastic search procedure, known as simulated annealing⁵ to identify the best atomic model or models. We discuss the potential of the method for structure determination and refinement, using results obtained for several known inorganic structures, and by the determination of a previously unknown structure. Although the approach is limited to the case of inorganic compounds, it is nevertheless very general, and would apply to any crystalline structure provided that the principles governing the architecture of the solid can be properly described.

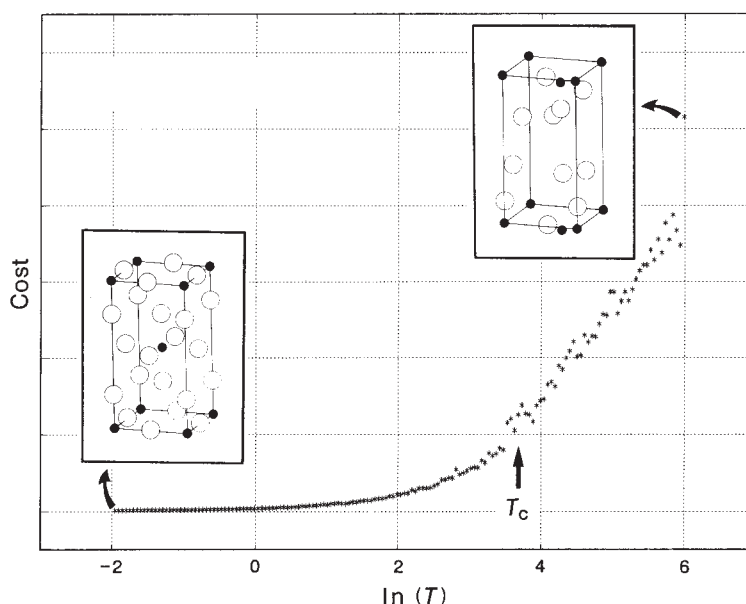
The structures of almost all known inorganic compounds are

consistent with the simple crystal chemistry principles put forward long ago⁴. Although these rules have been used extensively to assess the exactness and/or accuracy of newly determined structures⁶, their use to predict atomic-scale structural arrangements has been very limited. Recently Brown⁷ has developed a two-step method: (1) by trial and error, a three-dimensional model is built of the strongly bonded complexes, chosen using the nature of the elements; (2) the geometry is refined using a distance-least-squares (DLS) refinement⁸. The first step cannot easily be automated and must for the moment be performed by an expert operator.

The method described here, which focuses on ionic compounds, uses a different approach: the reasonableness of an ionic arrangement in an experimentally known unit cell is described by a 'cost' function which quantifies its departure from a set of building rules. To avoid getting trapped in local minima, this cost function is globally minimized by simulated annealing⁵: starting from a randomly generated arrangement and very slack constraints this algorithm progressively enforces the rules until a (hopefully) minimal-cost configuration is obtained. The only intervention required is the input of information (chemical formula, cell dimensions and valence-bond parameters) and control of the annealing program, which defines how rapidly the rules are enforced. This method deliberately ignores symmetry constraints (except the shape and size of the simulation box) and, at variance with most methods for structure modelling, treats all ions in the unit cell as independent species. A related technique of crystal structure determination, based also on simulated annealing but applied to zeolites and using a different cost function, has been recently described⁹.

The details of simulated annealing have been described in a number of reviews (see, for example, ref. 10). Briefly, four items are required to apply the algorithm: (1) a description of the system, that is, a set of parameters describing the size and shape of the unit cell, and the bond-valence characteristics of the ionic species. The initial coordinates are normally produced by a random number generator; (2) a cost (or energy) function—a scalar function that reduces the objective of the optimization to a single number; (3) a procedure to explore the configuration space of the system—this is achieved by a Monte Carlo technique using Metropolis importance sampling¹¹. Here, temperature is the control parameter; (4) a cooling (or annealing) schedule—the simulation starts at high temperature where almost all moves are accepted (liquid state) and several moves are attempted until

FIG. 1 Cost function E as a function of the logarithm of temperature during the annealing of NbF_4 structure. Bond-valence parameters: $r_0 = 1.96 \text{ \AA}$, $\rho = 0.27 \text{ \AA}$. Simulated-annealing parameters: initial 'temperature' $T_0 = 400$ (arbitrary units); cooling rate $\alpha = 0.95$; maximum move allowed $\delta_0 = 0.3 \text{ \AA}$ (initial), $\delta_f = 0.002 \text{ \AA}$ (final); δ was regularly updated to keep acceptance rate above 30%. To improve the statistics of the maps of the radial distribution functions the simulation was performed with 5,000 Monte Carlo cycles per temperature but convergence to the correct structure can be achieved with only 400 cycles per temperature. The weighting factor λ between the two terms of the cost function, calculated from the high-temperature part of the simulation, was ~ 0.85 . Inset: structural arrangement of NbF_4 at the beginning of the simulation (right) and as obtained by simulated annealing (left).



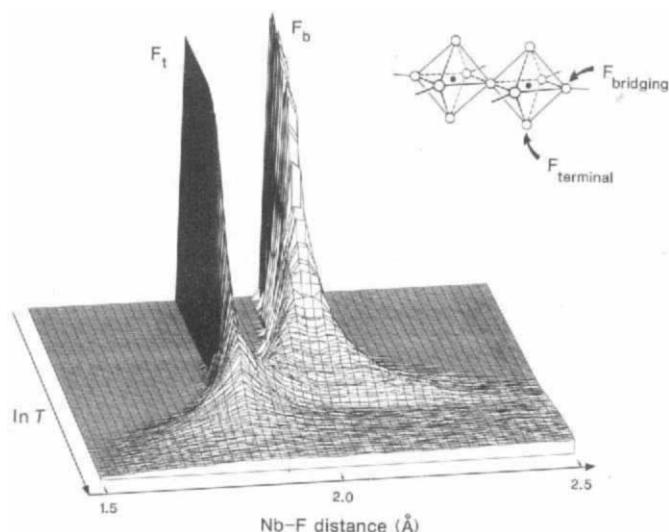


FIG. 2 Evolution of Nb-F radial distribution function in NbF_4 during annealing from 'molten' to crystalline state. The strong (short) Nb-F_{terminal} bond freezes at higher temperature than does the weaker (long) Nb-F_{bridging} bond.

the system reaches thermal equilibrium; the temperature is then reduced and the process is repeated until the system becomes frozen. We used a simple annealing program⁵ defined by the logarithmic law $T_{t+1} = \alpha T_t$ with $0.8 < \alpha < 1$.

We have used the electrostatic valence principle⁴ in its bond valence/bond length formulation⁶ to generate the cost function. The first term is defined as the overall departure from this rule, that is, the overall bond-valence unbalance of the configuration:

$$E_{\text{valence}} = \sum_i^{\text{cell}} \left| q_i - \sum_{j=1}^{\text{CN}_i} S_{ij} \right|$$

where q_i is the valence (formal charge) of ion i , CN_i its coordination number and S_{ij} the valence (strength) of bond ij ; S_{ij} is linked to interatomic separation r_{ij} by an usual empirical expression of the form $S_{ij} = \exp[(r_0 - r_{ij})/B]$ where r_0 and B are constants for a particular ion pair⁶. Coordination numbers are defined by applying a spherical cutoff to exclude from the second sum all neighbours j giving a contribution S_{ij} smaller than an arbitrary threshold (typically $0.02|q_i|$). In contrast to most energy functions used in structure modelling, the above expression does not refer to single pair interactions but involves the complete coordination sphere of every atom in the structure. This rule of local equilibrium controls the coordination of cations by anions and vice versa but does not restrict cation-cation or anion-anion packing. An elementary but not general method to constrain these two packings is through an electrostatic-like interaction of the form

$$E_{\text{electr}} = \frac{1}{2} \sum_{i,i'} \frac{q_i q_{i'}}{r_{ii'}}$$

where the indices i, i' cover all cation-cation and anion-anion pairs in the unit cell (using the minimum-image convention for truncation of the potential). Introducing a coefficient λ to express the relative cost of the two contributions, we obtain the total cost function $E = (1 - \lambda)E_{\text{valence}} + \lambda E_{\text{electr}}$. The choice of λ is rather critical for many structural types; we found that it can be conveniently estimated during the early stages of the simulation from the variance V of the two terms of the cost function: $\lambda = V_{\text{valence}} / [V_{\text{valence}} + V_{\text{electr}}]$ where $V_k = \langle E_k^2 \rangle - \langle E_k \rangle^2$.

The computing time depends both on the size of the simulated sample and on the annealing process. Starting from an arbitrary model, a typical run for a 10-atom structure (see NbF_4 below) consumes ~ 20 min of CPU (central processing unit) time on a VAX-8700.

Figures 1 and 2 show the performance of the method in the case of a simple compound, NbF_4 . The crystal structure consists of a tetragonal stacking of layers of corner-sharing octahedra¹² (see inset in Fig. 1). Although of modest complexity (10 atoms per unit cell) it exhibits two topologically different fluorine atoms and illustrates the essential features of the method. The progress of the simulation can be followed by the energy (Fig. 1) or by the radial distribution functions (Figs 2, 3). At the beginning of the simulation (high temperature), almost all configurations are allowed: the average cost function is high and the partial radial distribution functions are structureless. On cooling, short-range ordering progressively builds up and broad peaks appear which rapidly become narrower as the system freezes into a configuration that corresponds closely to the experimentally observed structure: the 12 independent Nb-F distances of the simulated structure differ by ≤ 0.04 Å from the two experimental values. An increase in the variance of the cost function signals the onset of the 'crystallization' (Fig. 4). It is interesting to note that when non-optimized values of the weighting factor λ are used or when the cooling is not properly performed, a molecular structure consisting of a packing of NbF_4 tetrahedra (similar to the structure of SnI_4) may be obtained.

The algorithm has been tested successfully on a variety of simple problems including three-dimensional structures (rock salt, rutile, fluorite, perovskite, BaSiF_6) and layer structures (KAlF_4 , K_2NiF_4). The structure of the first dirutile compound LiCoF_4 was actually solved by this method¹³ and later confirmed by neutron powder diffraction¹⁴. The magnetic structure of this compound was also predicted from postulated exchange integrals, using a method¹⁵ similar to this one.

At present the method is not efficient enough for use in most practical problems of *ab initio* structure determination. The optimization of the cooling schedule for continuous problems is still largely unsolved, leading to prohibitive amounts of computing time for structures with a large number of atoms or with a complex cost landscape (large number of local minima). But most of the present limitations of the method result from the

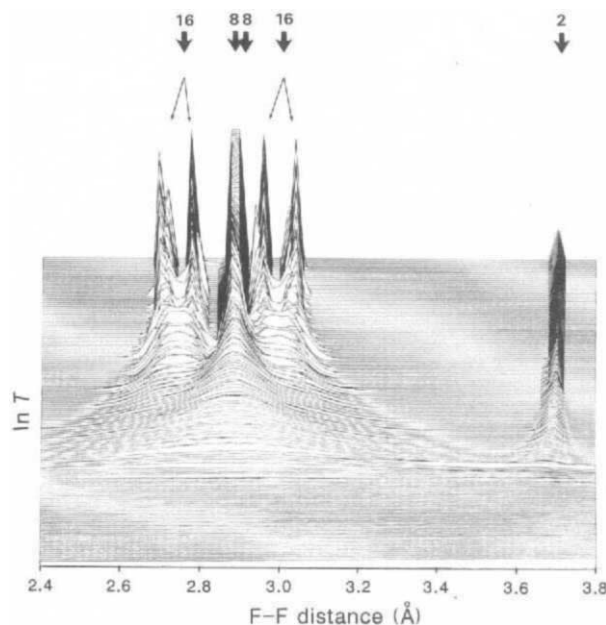


FIG. 3 Temperature-dependence of the F-F radial distribution function in NbF_4 . The arrows and numbers at the top indicate the experimentally observed distances and their multiplicities¹²; the topology obtained is correct but the limited accuracy of the bond valence parameters as well as small fluctuations in the bridging fluorine positions at low temperature split two of the equivalent F-F distances in two subgroups.

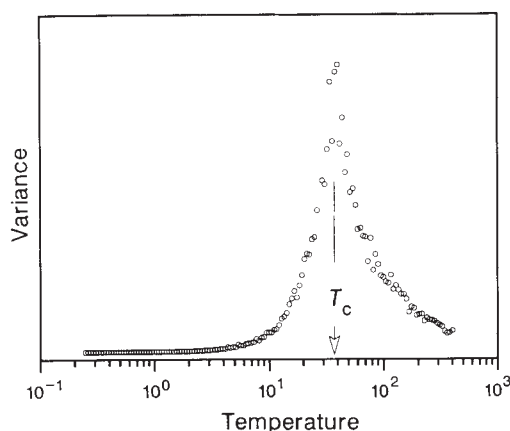


FIG. 4 Variance of the cost function of NbF_4 during the annealing process. The arrow indicates the 'crystallization' temperature.

simple cost function used. First, the 'naive' second term of the cost function we used performs well for ionic compounds that can be described as a close packing of spherical atoms but fails when applied to cations that form strongly directional bonds. For the same reason the cost function is not suited to structures exhibiting face-sharing polyhedra with highly charged cations (such as $\alpha\text{-Al}_2\text{O}_3$). Second, within the accuracy of the valence-bond rule (and parameters), different topological configurations are sometimes obtained for some compounds. This emphasizes the weakness of current empirical expressions used to correlate bond valence and bond lengths: their accuracy is adequate to check the validity of an experimentally determined atomic arrangement⁶ but we found that it is not always sufficient for reliable *ab initio* structure prediction. Other expressions such as that recently introduced by Hoppe *et al.*¹⁶ to calculate charge distributions in crystals may provide interesting alternatives. Another fruitful approach would be to explore the apparent similarity¹⁷ of the bond valence network to electric circuits and introduce the analogue of Kirchhoff's voltage law in the cost function. This ring sum rule, advocated by O'Keeffe¹⁸, provides further constraints which could complement or even replace the electrostatic term now used in the cost function.

Despite the current limitations, the results mentioned above demonstrate that the simulated annealing approach to structure prediction is a promising method for exploring the possible arrangements that are compatible with a set of prescribed building rules within the limits of a given unit cell. As such, the method is of general applicability: the challenge is to conjecture the empirical or semi-empirical rules that govern the architecture of the solids. □

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Phase transition in polymer gels induced by visible light

Atsushi Suzuki* & Toyochi Tanaka

Department of Physics and Center for Materials Science and Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

* Permanent address: Department of Materials Science, Yokohama National University, Hodogaya-ku, Yokohama 240, Japan

PHASE transitions and critical phenomena in polymer gels have attracted much attention because of their scientific interest and technological significance^{1–3}. Phase transitions accompanied by a reversible, discontinuous volume change as large as several hundred times, in response to infinitesimal changes in environmental conditions, have been observed universally in gels made of synthetic and natural polymers^{1–9}. Phase transitions have been induced in gels by varying temperature, solvent composition, pH, ionic composition and a small electric field¹⁰. Recently, gels sensitive to ultraviolet light were also reported¹¹. The ultraviolet light initiates an ionization reaction in the gel, creating internal osmotic pressure which induces swelling. In the absence of this light, the equilibrium tends towards the neutral polymer system and the gel collapses. This transition process is slow, as it depends on the photochemical ionization and subsequent recombination of ions, and it is technologically desirable that the transition be induced by faster mechanisms. Also, visible light is less harmful and more abundant than ultraviolet in sunlight. Hence we now report the phase transition of gels induced by visible light, where the transition mechanism is due only to the direct heating of the network polymers by light, which is an extremely fast process. Such systems might be used as photoresponsive artificial muscles, switches and memory devices.

Gels containing *N*-isopropylacrylamide (main constituent) and the light-sensitive chromophore, trisodium salt of copper chlorophyllin (Fig. 1), were formed by free-radical copolymerization as follows: 7.8 g of recrystallized *N*-isopropylacrylamide (Kodak), 0.67 g of *N,N'*-methylenebisacrylamide (cross-linker, Bio-Rad), 0.72 g of chlorophyllin (Aldrich) and 240 μl of tetramethylethylenediamine (accelerator, Bio-Rad) were dissolved in 100 ml of degassed water at 0 °C, to which 0.2 g of ammonium persulphate (Mallinckrodt) was added to initiate the polymerization. Capillaries of $\sim 100\text{-}\mu\text{m}$ inner diameter were

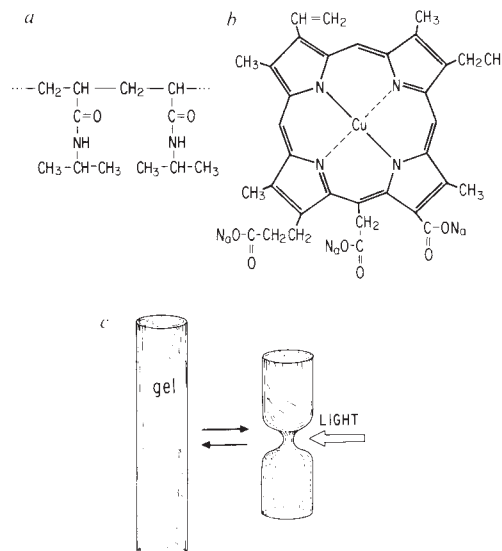


FIG. 1 Chemical structures of *N*-isopropylacrylamide (a) and trisodium salt of copper chlorophyllin (b). Chlorophyllin has a double bond that can be covalently connected to the polymer network. c, The collapse of the gel under illumination.

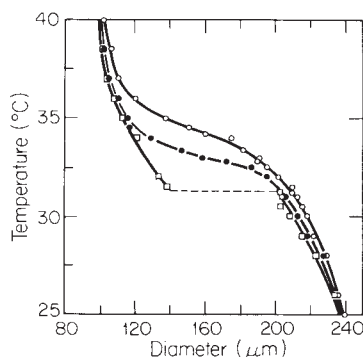


FIG. 2 Diameter of *N*-isopropylacrylamide/chlorophyllin copolymer gel as a function of temperature. Open circles, $I=0$ mW; solid circles, $I=60$ mW; squares, $I=120$ mW 488-nm radiation.

inserted into the solution. After gelation, the cylindrical samples were removed from the capillaries and dialysed extensively with distilled deionized water. A gel sample was then immersed in water (pH 5.8) or a NaOH solution (pH 11.9) and sealed in a rectangular glass microcapillary, the temperature of which was regulated to within ± 0.1 °C. Argon-ion laser radiation at 488-nm was used, and the light intensity at the gel was varied from 0 to 150 mW using a polarizer. The incident beam, gaussian diameter ~ 7 mm, was focused to 20 μm (focal depth 0.8 μm) at the sample using a lens of 19-cm focal length. The size and shape of the gel were then monitored and analysed using an image processor (Model C1966, Hamamatsu Photonics).

Figure 2 shows the equilibrium volume of the gel at pH 11.9 as a function of temperature for three values of light intensity. In the absence of illumination, the gel underwent a sharp but continuous volume change at ~ 35 °C. At a light intensity of 60 mW, the volume change became more pronounced and the 'bulk' equilibrium transition temperature was lowered to 33 °C. At an intensity of 120 mW the gel showed a discontinuous volume transition at 31.5 °C. The data in Fig. 2 show that the light-sensitive polymer gel is swollen in the absence of irradiation but collapses when illuminated with light of visible wavelength. Light shrank the gel over the entire temperature range, but the largest effect was seen in the transition region. These features

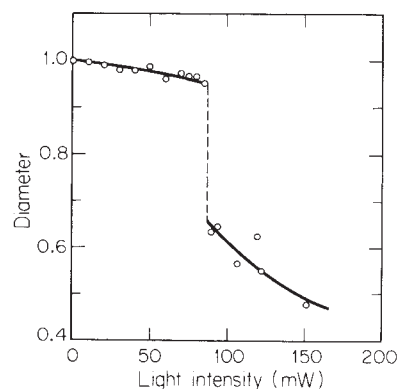


FIG. 3 Diameter of *N*-isopropylacrylamide/chlorophyllin copolymer gel as a function of intensity at 31.5 °C. The data were taken on the gels with the same composition but with an initial diameter different from the one used in Fig. 2.

were observed at both pHs. The results indicate that at an appropriate 'bulk' equilibrium temperature the gels should undergo a discontinuous transition when the light intensity is varied, as we observed when we fixed the temperature at 31.5 °C and varied the intensity from 0 to 150 mW (Fig. 3). The light intensity at the transition varies from gel to gel, as seen in the difference between Figs 2 and 3. The differences in the ratios of gel and beam diameters, or in the bleaching conditions may be responsible for the variation.

Two interesting features of the light-induced phase transition are that irradiation causes the originally continuous transition to become discontinuous and causes the transition temperature to be lowered. These can be explained using the Flory-Huggins equation of state. The swelling curve given by the condition of zero osmotic pressure for the gel is^{8,12}

$$\frac{1}{T} = \frac{\Delta S}{\Delta H} + \frac{k}{\Delta H} \left[\frac{v_1 v_c}{N \phi^2} \left\{ (2f+1) \left(\frac{\phi}{\phi_0} \right) - 2 \left(\frac{\phi}{\phi_0} \right)^{1/3} \right\} - \frac{2}{\phi} - \frac{2 \ln(1-\phi)}{\phi^2} \right] \quad (1)$$

where T is the absolute temperature, ΔS and ΔH are the entropy and enthalpy of polymer-polymer contact, k is the Boltzmann

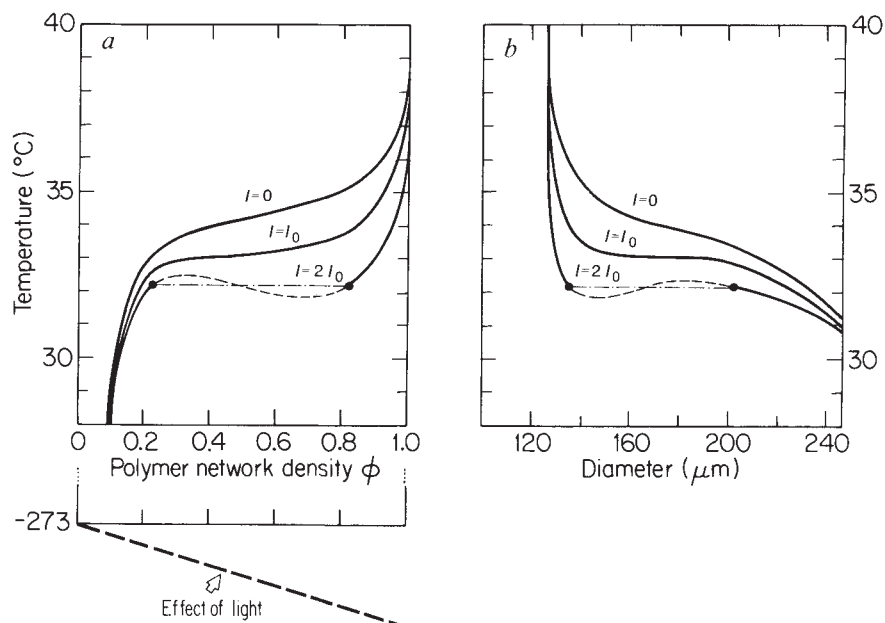


FIG. 4 *a*, Theoretical swelling curves of a gel for various light intensities, constructed using the equations in the text. The effect of illumination is to add a linear distortion of negative slope in proportion to the polymer density, *b*, Theoretical swelling curves as in *a* except that the horizontal axis is reduced diameter $d = d_0(\phi_0/\phi)^{1/3}$.

constant, N is Avogadro's number, v_1 is the molar volume of water, ν_e is the total number of the effective polymer chains in the gel, ϕ is the polymer network density, ϕ_0 is the density when polymers are in the random-walk configuration, and f is the number of counter-ions per chain. For N -isopropylacrylamide gels the interaction parameters ΔS and ΔH are both negative.

For simplicity we rewrite equation (1) as

$$T = T_{\text{gel}}(\phi) \quad (2)$$

This function increases monotonically with network density ϕ for small ionization f of carboxyl groups of chlorophyllin. When the gel is irradiated, the chromophore absorbs the light, which is then dissipated locally as heat by radiationless transitions, increasing the 'local' temperature of the polymer. The temperature increment should be proportional to the light intensity and the chromophore concentration, and therefore to the polymer network density ϕ . Thus

$$T = T_0 + \alpha I \phi \quad (3)$$

where T_0 is the ambient temperature, I is the light intensity, and α is a constant, $\sim 0.6^\circ\text{C}$ at $I = 100\text{ mW}$ (Y. Li, personal communication). Combining equations (2) and (3)

$$T_0 = T_{\text{gel}}(\phi) - \alpha I \phi \quad (4)$$

As illustrated in Fig. 4, the effect of the second term in equation (4) is to bring the gel state into the unstable region in the $T_0 - \phi$ space, inducing a discontinuous transition and to lower the transition temperature. Theoretical swelling curves and the contribution by light ($+\alpha I \phi$) to them are shown in Fig. 4. Because thermal diffusion is much faster than collective diffusion of the gel network, the swelling and shrinking process of the gel in response to light is governed only by the motion of the polymer network. For $1\text{-}\mu\text{m}$ -diameter gels the response time^{13,14} is expected to be $\sim 5\text{ ms}$. □

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Temperature and rainfall estimates for the past 40,000 years in equatorial Africa

R. Bonnefille*, J. C. Roeland* & J. Guiot†

* Laboratoire de Géologie du Quaternaire, CNRS Luminy, 13288 Marseille, France

† Botanique Historique et Palynologie, URA 1152, CNRS, Faculté St Jérôme, 13397 Marseille, France

THERE has been considerable debate about the magnitude of the decrease in temperature^{1,2} and the change in precipitation³ in the African tropics during the last glacial period. With the advent of fossil pollen studies in equatorial regions, it is now generally agreed that the temperature did decrease at this time in tropical regions⁴, but the magnitude of the temperature fluctuations and discrepancies between the continental⁵ and marine⁶ temperature records have yet to be resolved⁷. Here we present new quantitative estimates of temperature and precipitation using a multivariate analysis⁸ of pollen time-series data from peat deposits in Burundi for the past 40,000 years⁹. For the last glacial period, our estimate of a temperature decrease of $4 \pm 2^\circ\text{C}$ is less than those (ranging from 5 to 8°C) derived from snow-line and tree-line records^{5,7}. Model simulations⁷ indicate that the snow-line and tree-line estimates (from high-elevation sites at $\sim 4,000\text{ m}$ above sea level) are incompatible with the marine temperature record. Our lower estimate from a site of intermediate elevation may help resolve the differences between these records. We also estimate that the mean annual rainfall decreased by 30% during the last glacial period, in agreement with the rainfall history inferred from lake level fluctuations¹⁰.

Pollen data contains climate information which can be extracted using a variety of statistical techniques^{11,12}. Although the response of vegetation to climate needs further investigation¹³, pollen data have already provided successful quantitative estimates of climate parameters for temperate regions in North America¹², India¹⁴ and Europe^{15,16}. Here we focus on a fossil sequence recently obtained in the mountains of the Zaire/Nile

divide in Burundi⁹, which provides new data for the region south of the Equator¹⁷. Our statistical procedure has been described in detail elsewhere¹⁸.

The modern data set that we used is from East Central Africa between latitude 4°S and 12°N and longitude 28° and 42°E ¹⁹. It contains pollen samples from 356 sites that cover most plant communities from desert to subalpine grasslands and includes all types of forests²⁰. Mean annual rainfalls range from 200 to 2,000 mm (ref. 21). The decreasing temperature gradient is registered by the change with altitude of the vegetation on high mountains²² (Fig. 1).

For meteorological data, we compiled means of the modern measured values (normals) for about thirty years²³. To adjust data from the meteorological stations to the modern pollen sampling sites, an interpolation of temperature and precipitation values was carried out when necessary, according to the rules and procedure previously described⁸. Although the seasonal pattern linked to the movements of the Inter Tropical Convergence Zone is important in the tropics²⁴, we have focused on reconstructing the mean annual climate and have used the mean annual temperature and rainfall. The reconstruction of a palaeoclimate from pollen data relies on the assumption that climate is the main factor forcing changes in the vegetation and therefore pollen frequencies¹³. This is a subject of some debate, but we consider it to be valid for the 100-300 yr resolution of the fossil sequence.

Multiple regression shows that the modern-pollen database contains a significant climate signal: the correlation of estimated values extracted from pollen data to observed meteorological data was 0.90 for temperature and 0.80 for rainfall¹⁹. Although the regression method has been successfully applied to pollen data from temperate regions^{11,12,16}, it has gradually been replaced because it does not separate the different components of the climate signal. Best-analogues methods, such as surface response²⁵ and palaeobioclimate analogues¹⁵ have now been shown to yield plausible palaeoclimate estimates.

The analogue approach consists of looking for modern pollen data similar to the fossil ones and of equating the appropriate modern climate to the palaeoclimate. The statistical distance is a measure of the difference between the modern and the fossil-pollen assemblages¹⁷. In our palaeobioclimate approach, this

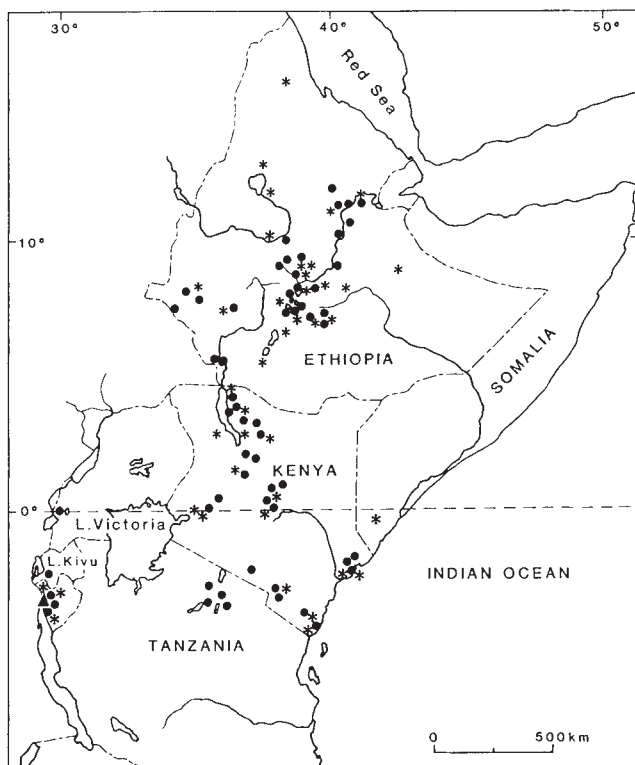


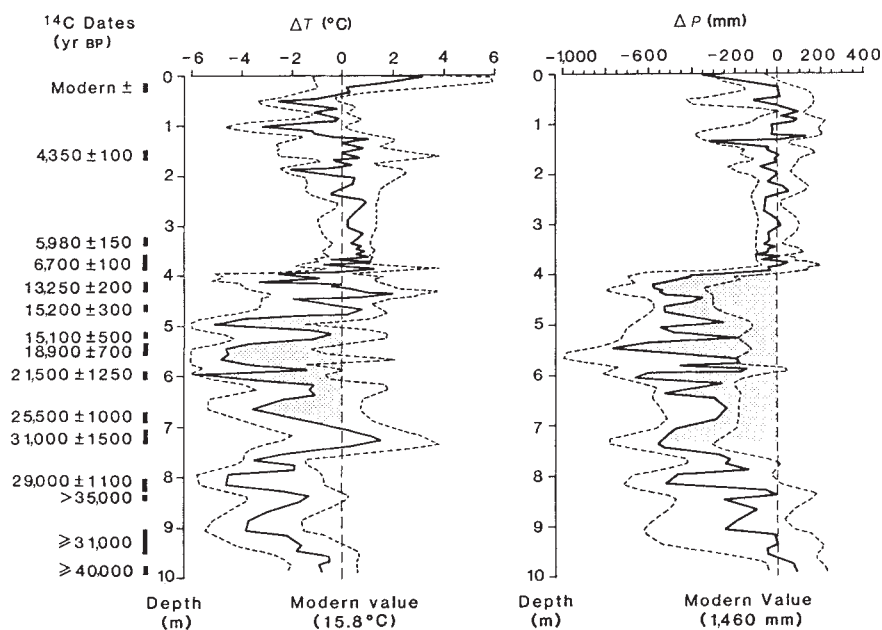
FIG. 1 Location map of the modern pollen samples used for reconstruction of the palaeoclimate*. Meteorological station sites; ●, Modern pollen; ▲, core site.

has two factors. First, the pollen percentages of both the modern and the fossil data are transformed into logarithms to smooth the variations of the over-represented pollen taxa. Second, a statistical weight is attached to each pollen taxa. If we had used modern pollen data to calculate these weights, they could have been biased owing to human disturbance of the relation between pollen frequencies and climate, even if much-disturbed vegetation had been avoided by selective sampling. Unlike other studies, we calculate these weights using a time-series analysis of the fossil sequence. The weights are given by the first eigenvec-

tor of the cross-correlation matrix²⁶ between the different pollen taxa of the fossil sequence. As in the case of the first principal component of the standard correlations between taxa, this first eigenvector reflects the major variations of the fossil pollen data. Moreover, we use cross-correlations at lag 1 (that is, correlations between successive depth levels) to emphasize those taxa with a coherent temporal behaviour. The first eigenvector is then presumed to be a good monitor of the evolution of climate.

In the time-series analysis of this fossil sequence, the first eigenvector accounts for >60% of the sum of the squared

FIG. 2 Reconstructed mean annual temperature and rainfall at Kashiru, 3°28' S, 29°34' E, Burundi, expressed as deviation from the modern value. The dashed lines represent the mean of lower and upper standard deviations.



autocorrelations of the pollen percentages of 70 selected pollen taxa. Table 1 lists those that dominate the first eigenvector and the statistical distance between the modern and the fossil pollen spectra.

The reconstructed temperature and rainfall estimates are obtained by averaging the values of the best 20 modern analogues, weighted by the inverse of the squared distance. These calculations were done for each of the pollen assemblages at individual stratigraphic levels of the fossil sequence. The modern analogues with a temperature less than this average provide the lower standard error and the analogues with a greater temperature provide the upper one. This results in an asymmetrical confidence interval around the mean reconstructed climate parameters. The mean errors are found to be 2.2°C for temperature and 200 mm for precipitation, both with a probability of 0.70. The curves in Fig. 2 have been smoothed by a three-point digital filter to avoid fluctuations limited to one or two samples.

The quality of the reconstructions was tested by estimating the climate parameters at the modern pollen samples sites using the same modern analogues and the weights of pollen taxa previously calculated from the fossil sequence. The correlations between the estimated and measured meteorological values, have coefficients of 0.94 for temperature and 0.85 for precipitation, indicating that the reconstructions are soundly based. The statistical significance of this test is high, especially in light of the fact that the weights were calculated from the fossil data, not from the modern pollen spectra.

Figure 2 gives the mean annual temperature and rainfall curves reconstructed from the 40-kyr pollen sequence from a site at 2,240 m altitude in the equatorial highlands of Burundi. They are presented as a function of depth with the ^{14}C chronology. Pollen counts for 54 more stratigraphic levels have been added to the 78 data published earlier⁹ and the average time resolution is therefore a few hundred years. Despite a probable unexplained hiatus in the late glacial and early Holocene, the Kashiru Swamp is one of the few pollen sites in Africa with records extending back 40,000 years.

Except for one study²⁷, which used a different statistical approach, we are not aware of other quantitative estimates of palaeoprecipitation for tropical Africa. We cannot discuss this record in detail until the mean annual rainfall has been reconstructed for more complete Holocene sequences. The strong aridity trend during the last glacial period needs no further comment as it is well documented by the geological record^{10,17,28}.

To our knowledge, Figure 2 shows the first continuous palaeotemperature record for the tropics. During the past few thousand years, the average of the reconstructed temperature curve is close to the modern values. The over-estimation of the top-most sample is due to the recent deforestation around the site. The record indicates an average temperature decrease of $4\pm 2^{\circ}\text{C}$ for the glacial period between 30 and 13 kyr BP. Moreover, the curve seems to be consistent with the evolution of past vegetation indicated by the same fossil pollen record⁹. Before 30 kyr, the cooling is consistent with the occurrence of montane conifer forest on the Burundi highlands ($>2,200\text{ m}$), which are now tropical rainforest. It also agrees with other pollen data from East Africa¹⁻³. The lowest temperature is recorded between 25 and 15 kyr, when the presence of the tree-line and afroalpine grassland at the Kashiru site imply that the mountain vegetation belts had descended by 1,000–1,500 m. The magnitude of cooling in the tropics during the last glacial period is a subject of active discussion. Estimates based on the depression of tree-lines and snow-lines in the high mountains of East Africa, New Guinea and South America indicate a glacial cooling of $5-8^{\circ}\text{C}$ ^{5,7}, which appears to be incompatible both with CLIMAP estimates of sea surface temperature and with the temperature in the free atmosphere simulated by the current generation of general circulation models⁷. The Kashiru site is situated at an intermediate elevation. Our estimate of the last

TABLE 1 Pollen taxa with the largest loading in the palaeobioclimate operator

Taxa	Statistical distance
Gramineae	+0.38
<i>Anthospermum</i> (Rubiaceae)	+0.26
Ericaceae	+0.22
<i>Podocarpus</i> (Podocarpaceae)	-0.13
<i>Alchemilla</i> (Rosaceae)	-0.15
Combretaceae	-0.16
<i>Hagenia abyssinica</i> (Rosaceae)	-0.16
<i>Polyscias fulva</i> (Araliaceae)	-0.19
<i>Vernoniae</i> (Compositae)	-0.20
<i>Hypericum</i> (Guttiferae)	-0.20
<i>Tubuliflorae</i> (Compositae)	-0.26
Urticaceae	-0.27
<i>Alchornea</i> (Euphorbiaceae)	-0.31
<i>Macaranga</i> (Euphorbiaceae)	-0.34

glacial cooling, $4\pm 2^{\circ}\text{C}$, is a more conservative value than the estimate of $5-8^{\circ}\text{C}$ made at the same site⁹, which was based on the assumption that the lapse rate was the same as the present value, $5-6^{\circ}\text{C km}^{-1}$. This is not surprising because displacements of vegetation belts, although difficult to evaluate, depend not only on cooling but also involve precipitation. Our results suggest that the discrepancy between continental and oceanic glacial cooling lies in a failure to distinguish properly between the effects of changes in precipitation and temperature. The curve in Fig. 2 indicates that the optimal time for glacial advance in East Africa (which has not been dated so far) was $\sim 21,500$ years ago, when the climate was cold but relatively moist. Our work also indicates that the inferred continental glacial cooling from other tropical regions of the world²⁹⁻³¹ may have been over-estimated. Discrepancy between terrestrial temperature decrease for New Guinea and South America and adjacent sea surface temperature might be strongly reduced by further quantitative estimates such as those we have obtained for equatorial Africa. □

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Polar ice ablation rates measured using *in situ* cosmogenic ^{14}C

D. Lal*, A. J. T. Jull†, D. J. Donahue‡, D. Burtner‡
& K. Nishiizumi§

* Scripps Institution of Oceanography, La Jolla, California 92093, USA

† NSF Accelerator Facility for Radioisotope Analysis,

University of Arizona, Tucson, Arizona 85721, USA

‡ Isotope Laboratory, Scripps Institution of Oceanography, La Jolla, California 92093, USA

§ Department of Chemistry, University of California, San Diego, La Jolla, California 92093, USA

THE first suggestion that appreciable ^{14}C might be produced *in situ* in polar ice was made by Fireman and Norris¹, who studied ^{14}C in CO_2 extracted from both accumulation and ablation samples. In some ablation samples they observed ^{14}C activities between four and six times higher than those expected due to trapped atmospheric CO_2 . Here we report the detection of an unambiguous signal of *in situ* cosmogenic ^{14}C in ice samples from two ablation sites in the Antarctic. The ^{14}C is produced mainly by nuclear spallations of oxygen in ice. The observed concentration of ^{14}C in ablation ice samples is $1\text{--}3 \times 10^3$ atom per g ice—three orders of magnitude higher than expected from the amount of trapped atmospheric CO_2 in this ice. The *in situ* ^{14}C has a unique signature: about 60% exists as ^{14}CO and the remainder as $^{14}\text{CO}_2$. This result is consistent with that expected from studies of artificially produced ^{14}C in solid targets. The ^{14}C concentration is found to decrease with depth as expected for *in situ* production. The calculated model ablation rates are found to be 5.8 ± 0.7 and $7.6 \pm 0.8 \text{ cm yr}^{-1}$ at two sites from the Allan Hills main ice field, in agreement with rates determined by the stake method. Our work indicates that the ^{14}C age of accumulation ice based on trapped (atmospheric) CO_2 would be an underestimate of the true age, if a correction is not made for *in situ* produced $^{14}\text{CO}_2$. This can be done easily because the ^{14}C activities of both the CO and CO_2 phases, as well as the trapped CO_2 concentration, can be measured.

The ice samples were collected during the 1987–1988 field season at Allan Hills main ice field by D. Burtner. The sample locations are Cul-de-Sac ($76^\circ 46' \text{ S}$; $159^\circ 26' \text{ E}$) and ($76^\circ 43' \text{ S}$, $159^\circ 22' \text{ E}$), near station 10 of the triangulation chain by Nishio and Annestad^{2,3}. The elevation² of the sites is $\sim 2,000 \text{ m}$. Blocks of $\sim 45 \times 45 \times 60 \text{ cm}$ (depth) were cut and returned whole to the laboratory and stored at -20°C until use.

Extraction of ^{14}C activity in the ice samples was carried out using a clean vacuum system containing no mercury or teflon. The pumping system consisted of a roughing pump and an oil diffusion pump using a silicone oil of ultra-low vapour pressure. Circulation of gases in the vacuum system was accomplished

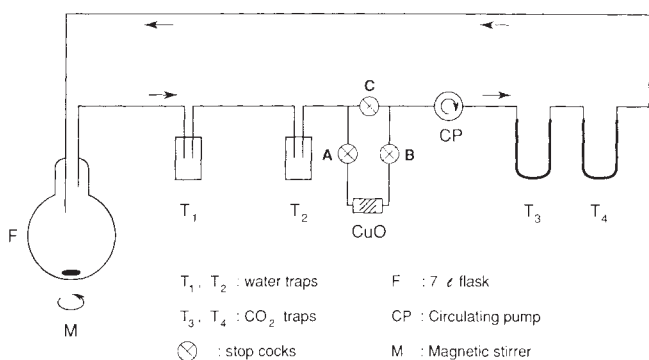


FIG. 1 A schematic of the vacuum line used for extraction of ^{14}CO and $^{14}\text{CO}_2$ from ice samples.

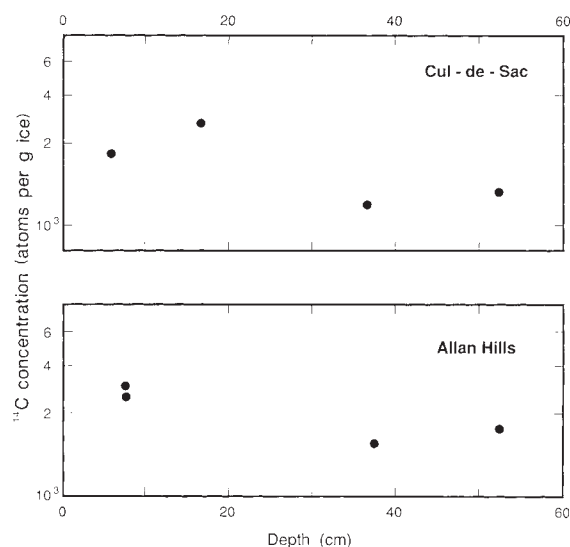


FIG. 2 The measured total ^{14}C concentrations in ice samples, corrected for line blanks, in the CO_2 and CO phases plotted as a function of depth for the Allan Hills main ice field Station 10 and the Cul-de-Sac ice samples.

using a metal-bellows circulating pump (Metal Bellows Corp., Model MB-158) that delivered a pressure head of ~ 5 torr at a pressure of 20 torr.

Ice samples were crushed into small pieces and loaded into a 7-l spherical pyrex flask connected to the vacuum system (Fig. 1). The flask was cooled in a mixture of salt and ice and then pumped down. Carrier gases of ^{14}C -free CO and CO_2 ($1\text{--}2 \text{ cm}^3$) were added. The ice sample was then allowed to melt, and the melt water was cooled to 0°C keeping it well stirred. With stopcocks A and B closed, the circulating pump was then started and the CO_2 frozen out ($\sim 2\text{--}3 \text{ h}$). The residual gas was passed over conditioned CuO at 600°C for 1 h with stopcock C closed, and for a further 2 h while the CuO was allowed to cool to $<250^\circ \text{C}$. The CO_2 gas formed by oxidation of CO (the ' CO fraction') was then frozen out.

Early tests showed that an appreciable amount of the carrier CO_2 was not recovered. This was found to be because the pH of the ice was in the range 6–7, therefore 1 M HNO_3 was added lower its pH to ~ 2 . The yields of CO_2 were 30–90%. We assumed that the isotopic ratio of the gas recovered was representative of the total gas and that no exchange between CO_2 and CO had occurred.

The $^{14}\text{C}/^{12}\text{C}$ ratios of CO and CO_2 samples were determined at the Arizona Accelerator Facility. The results are presented in Table 1 along with the sample details. The concentrations of ^{14}C in CO_2 fraction were calculated assuming that the amount of trapped atmospheric CO_2 was 0.03 cm^3 per kg ice (ref. 4). The calculated values of ^{14}C concentrations have to be corrected for the line blanks. Based on two runs, we determined that the blanks for CO_2 and CO were 2.06 ± 0.14 and $0.32 \pm 0.12\%$ of modern carbon respectively. The errors are deduced from counting statistics only. Past experience shows that uncertainties in blank measurements are usually greater than the uncertainties in the counting statistics and are $\sim 25\%$ of the measured values. Here we used 2.0 ± 0.5 and $0.5 \pm 0.5\%$ modern for the CO_2 and CO blanks, respectively. As expected if the content in the blank resulted from atmospheric contamination, the CO blank is much smaller than the CO_2 blank. The blanks are much lower than the signals seen for the ice samples. The equivalent measured ^{14}C concentrations are 278 ± 70 and 40 ± 14 ^{14}C atom per g for CO_2 and CO blanks, respectively, assuming that the blank was extracted from 4 kg of ice; the actual quantity of ice varied

TABLE 1 ^{14}C in ablation ice samples

Sample code	Depth (cm)	Weight of ice (kg)	Carrier added (cm ³)		¹⁴ C activity (% modern)		¹⁴ C concentration (atom per g ice)*		Total
			CO ₂	CO	CO ₂	CO	CO ₂	CO	
Allan Hills Station 10									
ALH 2 (1) T	0-15	5.59	2.11	1.00	14.7±0.7	23.1±0.6	1,490±100	1,160±40	2,655±110
ALH 3 (1) T	0-15	5.22	1.79	1.76	12.5±0.25	20.1±0.33	1,130±60	1,910±60	3,035±85
ALH 2 (2) T	30-45	5.45	2.07	0.98	7.0±0.6	18.7±0.3	590±90	940±30	1,535±100
ALH 2 (2) B	45-60	4.65	2.03	0.97	8.02±0.2	16.3±0.2	810±70	940±30	1,755±80
Cul-de-sac									
CULDE 2 (1) T	0-11.5	4.72	1.84	1.92	8.38±0.24	9.51±0.2	770±70	1,060±60	1,830±90
CULDE 2 (1) B	11.5-22.9	4.45	1.80	1.83	7.89±0.19	14.4±0.2	740±70	1,640±60	2,380±90
CULDE 1 (2) T	30-45	6.25	2.02	0.95	7.28±0.17	15.5±1.6	540±50	650±70	1,190±90
CULDE 1 (2) B	40-60	6.11	1.96	0.92	8.41±0.2	15.5±0.5	650±50	650±30	1,300±65

* Corrected for line blanks of CO₂ and CO; see text.

from 4.7–6.1 kg (Table 1). The total blank-corrected ^{14}C concentrations in the ice samples for the two fractions after subtracting the respective line blanks are plotted in Fig. 2.

As seen in Table 1, the repeat measurement of the depth of 0–15 cm at Allan Hills shows a difference in ^{14}C concentrations in the CO fraction, much greater than that expected from quoted errors. Presumably this discrepancy occurred because of incomplete trapping of CO₂ during the first phase of the extraction. This would result in some ^{14}C in CO₂ being identified as ^{14}C in CO.

The ablation ice in the Allan Hills main ice field is expected to be quite 'old', >100,000 yr based on uranium-series dating method^{5,6}, which would be consistent with the old terrestrial age of meteorites⁷ found in the Allan Hills main ice field. The expected ^{14}C concentration in zero-age ice owing to trapped atmospheric CO₂ in the ice samples would be ~950 atom per g ice, taking the typical CO₂ concentration to be 0.03 cm³ CO₂ per kg ice (ref. 4). Assuming the age of the ice to be 50,000 yr (a lower limit), we expect the ^{14}C concentration owing to the atmospheric trapped CO₂ to be only ~2 atom ^{14}C per g ice. Similarly, we expect the residual *in situ* ^{14}C produced in the ice during its accumulation to be <2 atom ^{14}C per g ice. Thus the signals observed in the CO₂ and CO fractions are due nearly entirely to the ^{14}C produced *in situ* in the ice during its outcropping stage. The depth profiles of total ^{14}C concentrations (Fig. 2) are not inconsistent with the expected 1/e depth of ~170 cm (ref. 8) for *in situ* ^{14}C , taking the mean density of ice to be 0.9 g cm⁻³.

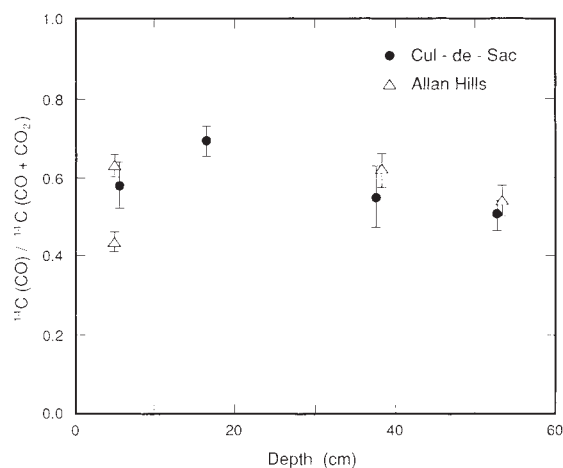


FIG. 3 The measured ratios of concentrations of ^{14}C in the ^{14}CO phase, relative to the total in $^{14}\text{CO}_2$ and ^{14}CO phases, plotted for each of the ice samples studied.

The mean fraction of ^{14}C in CO is 0.57 ± 0.08 compared to the total, for all the measurements (Fig. 3). This should represent primarily the fraction of *in situ* ^{14}C converted to ^{14}CO as the *in situ* produced ^{14}C atom comes to rest^{9,10}. In experiments conducted with 'hot' ^{11}C atoms, the ^{11}CO and $^{11}\text{CO}_2$ fractions were found to depend on the nature of the target in which ^{11}C is produced. For solid phase (solid CO₂ and NaHCO₃ targets), the yields were found to be about equal⁹, in good agreement with our result of ~0.6.

The *in situ* production rate of ^{14}C in ice by oxygen spallation has been estimated⁸. At geomagnetic latitudes $\geq 60^\circ$ and 2,000 m altitude this rate is estimated to be 84 atom g⁻¹ yr⁻¹. (There is an error in the *in situ* production rate of ^{14}C at 2 km altitude ($\lambda \geq 60^\circ$) given in Lal *et al.*⁸. Using the mean altitude–pressure relationship¹¹, the revised values for *in situ* ^{14}C production rates owing to spallations in oxygen in ice at 0, 1, 2, 3 and 5 km altitudes and geomagnetic latitudes $\lambda \geq 60^\circ$ are 15, 38, 84, 164 and 480 ^{14}C atom g⁻¹ yr⁻¹, respectively.) To this we add a small contribution (<2%) from thermal neutron capture in ^{14}N present in the ice as a constituent of the trapped 'air'. The total *in situ* production rate of ^{14}C in ice at Allan Hills (altitude 2 km and geomagnetic latitude ~60°) is estimated to be 85 atom per g ice yr⁻¹.

The concentration of ^{14}C produced *in situ* in Allan Hills quartz exposed at 2 km was found to be $(8.2 \pm 1) \times 10^5$ atom per g SiO₂ (ref. 12). Based on studies of *in situ* ^{10}Be and ^{26}Al in this sample¹³, the ^{14}C activity is in secular equilibrium with its production. This yields a production rate of 99 ± 12 atom ^{14}C per g quartz yr⁻¹. This result supports our estimate for production rate of ^{14}C in ice, but suggests that it may be an underestimate by ~20%.

We have determined the mean ablation rates for the ice samples based on the uniform ablation model⁸. This model predicts that the steady-state concentration at depth x (cm) from the surface owing to *in situ* production is

$$C(x) = \frac{P_0 e^{-\rho x / \Lambda}}{\lambda + \rho a / \Lambda} \quad (1)$$

where P_0 is the *in situ* production rate of ^{14}C , ρ (g cm⁻³) is the density of ice, Λ is the absorption mean free path (g cm⁻²) of cosmic rays in ice, 150 g cm⁻² (ref. 8), and a is the rate of ablation of ice (cm yr⁻¹). For given measured values of $C(x)$, the equation was solved numerically for the ablation rate a . We obtained 5.8 ± 0.7 and 7.6 ± 0.8 cm yr⁻¹ for station 10 and Cul-de-Sac respectively, in good agreement with the value of 3–9 cm yr⁻¹ measured^{2,3}, using stakes, for the Allan Hills main ice field. Annestad and Schultz¹⁴ obtained an average value of 4.5 cm yr⁻¹ for the past 10 yr and W. A. Cassidy (personal communication), a value of ~3.5 cm yr⁻¹ for 8-yr average (1978–1986) at a site 2 km northwest of Cul-de-Sac. If the ablation rate has varied significantly in the past, the model ablation rates of ~5.8 and 7.6 cm yr⁻¹ would correspond to the rates operative

during the period when the ice outcropped from a depth of ~ 2 –3 absorption mean free paths, that is, the past ~ 50 years. The *in situ* concentration in near-surface ice is attained mostly during this stage.

We did not make any corrections for the atmospheric 'trapped' ^{14}C component because these samples are 'old'. In younger samples, one can determine the absolute amount of the trapped $^{14}\text{CO}_2$ component by studying a depth profile of samples. The trapped component is expected to remain constant whereas the *in situ* component will show a depth dependence.

The potential application of *in situ* ^{14}C in accumulation ice have been discussed⁸. Our findings indicate that the *in situ* ^{14}C signal can be seen clearly even in rapidly accumulating ice. For ice accumulating at 2 km altitude in the polar regions, the expected *in situ* ^{14}C concentrations would attain values of $\sim 1.4 \times 10^4/s$ atoms g^{-1} , where s is the accumulation rate (cm yr^{-1}). Because $\sim 60\%$ of the *in situ* ^{14}C is converted to ^{14}CO , one can easily measure accumulation rates up to $\sim 50 \text{ cm yr}^{-1}$ in ~ 3 –5 kg ice. □

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Natural occurrence of silicon carbide in a diamondiferous kimberlite from Fuxian

Irene Leung*, Wenxiang Guo†, Irving Friedman‡ & Jim Gleason‡

* Department of Geology and Geography, Lehman College, City University of New York, Bronx, New York 10468, USA
† 701 Diamond Mine, Mengyin, Shandong, People's Republic of China
‡ US Geological Survey, Box 25046, Mail Stop 963, Denver, Colorado 80225, USA

CONSIDERABLE debate surrounds the existence of silicon carbide in nature, mostly owing to the problem of possible contamination by man-made SiC. Recently, Gurney¹ reviewed reports of rare SiC inclusions in diamonds, and noted that SiC can only be regarded as a probable rather than proven cogenetic mineral. Here we report our observation of clusters of SiC coexisting with diamond in a kimberlite from Fuxian, China. Macrocrysts of α -SiC are overgrown epitaxially by β -SiC, and both polymorphs are structurally well ordered. We have also measured the carbon isotope compositions of SiC and diamonds from Fuxian. We find that SiC is more enriched in ^{12}C than diamond by 20% relative to the PDB standard. Isotope fractionation might have occurred through an isotope exchange reaction in a common carbon reservoir. Silicon carbide may thus ultimately provide information on carbon cycling in the Earth's mantle.

Because other workers failed to find the SiC that Moissan³ claimed to have found in the Canyon Diablo meteorite², Mason³ suggested that Moissan's SiC might have come from the saw used to cut the meteorite. Reports^{4,5} of SiC containing data indicative of possible natural origins are rare, and only one paper⁶ has described α -SiC in some detail. Data on β -SiC reportedly found in the Green River Formation⁷ are very scanty. The only other reported occurrence of β -SiC, described as black spherules in α -SiC (polytype 15R)⁸, was based on an X-ray powder diffraction pattern. We believe, however, that this identification cannot be substantiated because the X-ray lines of β -SiC and type 15R overlap completely. The almost 50 samples reviewed by Lyakhovich⁹ and described in the original papers resemble artificial SiC (carborundum). Three common sources of contamination are the carborundum fragments used in polishing rock specimens, the drilling equipment bonded with euhedral carborundum crystals used in mining operations, and heavy minerals recovered from rivers polluted with industrial waste¹⁰.

Our specimens came from a kimberlite pipe emplaced in the Sino-Korean Craton near Fuxian, Liaoning (latitude, 39.63° N; longitude, 122.00° E). This pipe contains both gem-quality and industrial-grade diamonds. The concentrations of SiC vary from 0 to 0.15% of the host rock—in one instance, we crushed 20 g of kimberlite in an agate mortar, washed the rock fragments to discard the dust-sized particles, and recovered 30 mg of SiC by hand-picking under a binocular microscope. This technique was used throughout our investigation to avoid the use of heavy liquids and contaminations. We used a petrographic microscope to select crystals for analysis by SEM/EDX (scanning electron microscopy/energy-dispersive X-ray) techniques. A Princeton Gamma-Tech Si(Li) windowless detector was used so that the presence of light elements such as C, N, O and F could be verified directly. The solid inclusions and polytype structure were studied using a single-crystal X-ray precession camera. We also measured carbon isotope compositions of 20 mg of α -SiC and five samples of inclusion-free diamonds derived from the same kimberlite pipe.

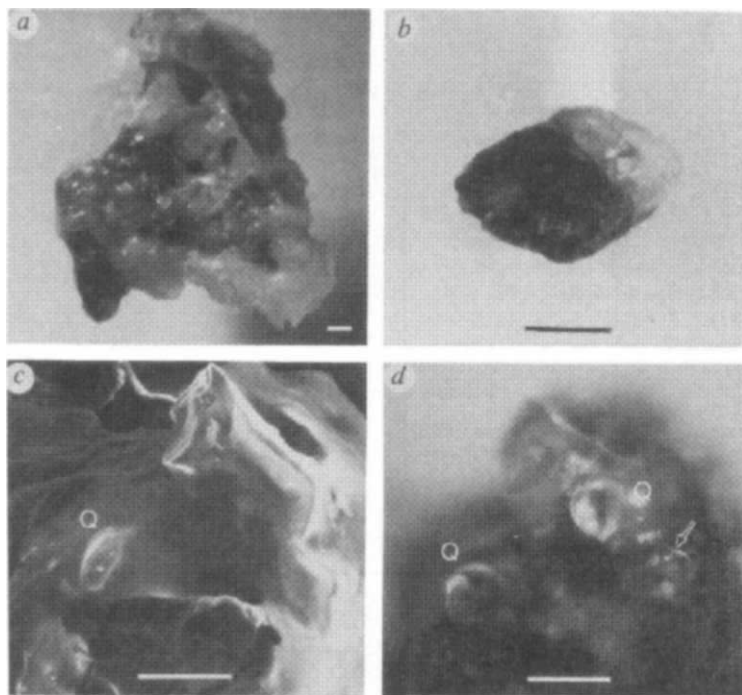
Large α -SiC crystals (up to 5 mm) are deep blue, but very small crystals often display colour-zoning. Plate-like crystals have a well developed {10 $\bar{1}$ 0} form and are elongated along the a -axis. Rounded resorbed morphologies are common. Crystal aggregates are frequently agglomerated by vesicular milky β -SiC (Fig. 1a), and transparent euhedral crystals of β -SiC often occur with the vesicular type (Fig. 1d). Intermingled with the β -SiC are spherical grains of quartz occurring as inclusions, protrusions and overgrowths (see Fig. 1c, d), that are invisible in SEM photographs when totally included by β -SiC (Fig. 1c). Many crystal faces are coated by a thin film of iridescent native Si, identified by SEM/EDX.

The zero-level a -axis X-ray precession photograph (Fig. 2a) of the dark crystal in Fig. 1b shows a well ordered 6H-polytype structure. The light side of the same specimen has a 3C structure (Fig. 2b) in which abnormal intensities of the 220 reflections are due to epitaxial growth in which the [110] _{β} and [10 $\bar{1}$ 0] _{α} layers are parallel. The 3C structure is also well ordered, as indicated by the discrete diffraction spots. By contrast, commercial SiC is generally composed only of α -SiC that contains numerous stacking faults and multiple polytypes¹¹.

Crystallographic data of quartz inclusions are derived from X-ray photographs. The reciprocal-lattice net shown in Fig. 2c may represent a crystal of either hexagonal or trigonal symmetry, as X-ray patterns are centrosymmetric. The lack of vertical and horizontal symmetry planes parallel to c^* and a^* , respectively (Fig. 2d), could indicate a $\bar{3}2$ point-group symmetry (trigonal), the symmetry of low quartz (α). But, only a few reflections lack mirror images (owing to extinction or variation in intensity), so the quartz inclusions are, in fact, pseudohexagonal, closely resembling the symmetry of high quartz (β).

As SiC can be synthesized only at very high temperatures, and because of its association with diamond-bearing kimberlite,

FIG. 1 Scanning electron micrographs of *a*, deep-blue α -SiC crystals overgrown by vesicular milky β -SiC; *b*, epitaxial growth of α -SiC (dark) and β -SiC (light), identified by their X-ray photographs (Fig. 2*a, b*); *c*, β -SiC with protruding quartz crystal (Q). Scale bars 100 μ m. *d*, Photomicrograph of the same area as *c*, showing two spherical quartz inclusions, which become visible in transmitted light. Arrow indicates euhedral β -SiC crystal. Scale bar 100 μ m.



a source region in the Earth's mantle is inferred. Appearance of β -SiC as overgrowths indicates that α -SiC phase must be formed first. Coexisting α -SiC and native Si constitute an assemblage that is stable in environments where the oxygen fugacity is more reduced than the Si-SiO₂ buffer, but we note that geological buffer systems may be highly pressure-dependent¹². The discovery of a cluster of SiC entrapped in a diamond from Fuxian¹³ indicates diamond-SiC paragenesis. Woermann and Rosenhauer¹⁴ estimated theoretically that an assemblage such as diamond + SiC + olivine + orthopyroxene may represent an extremely low redox state. To determine how reduced a chemical environment existed in the mantle when assemblages such as

diamond-SiC or diamond-SiC-Si were formed requires a knowledge of the reactants involved in their reaction paths.

Development of vesicular textures in β -SiC requires a gas-rich depressurized environment, which may be encountered during vesiculation of a kimberlitic magma enriched in CO₂. Its rapid ascent¹⁵ may quench and preserve the metastable 3C structure¹⁶ of β -SiC. The appearance of quartz as inclusions and protrusions in β -SiC indicates a cogenetic relation. In the presence of CO₂, native Si associated with α -SiC may undergo the reaction $\text{Si} + \text{CO}_2 \rightarrow \text{SiC} + \text{O}_2$ to form β -SiC. In the presence of excess Si, the reaction becomes $2\text{Si} + \text{CO}_2 \rightarrow \text{SiC} + \text{SiO}_2$, producing β -SiC and quartz. The pseudohexagonal symmetry of quartz may be the

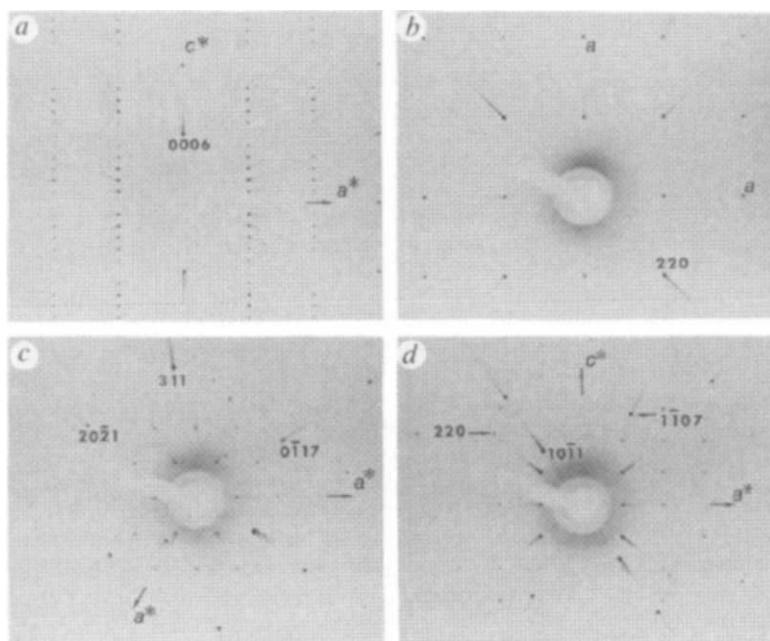


FIG. 2 Zero-level single-crystal X-ray precession photographs. *a*, α -SiC, 6H polytype, *a*-axis. *b*, β -SiC, 3C polytype, *a*-axis. *c*, Spherical quartz inclusion (near centre in Fig. 1*d*), *c*-axis. *d*, Same quartz inclusion, *a*-axis. Indexed extraneous reflections in both *c* and *d* are derived from α - and β -SiC. Mo K _{α} X-rays (generated using a 20-mA electron beam accelerated to 45,000 V) were used for all photographs. Exposure times, 2–4 days.

TABLE 1 Carbon isotope values of SiC and diamonds from Fuxian

Mineral	Number of crystals	Weight (mg)	Description	$\delta^{13}\text{C}(\text{‰})^*$
α -SiC	Numerous	20	blue	-24.0
Diamond	11	12	white, flawless	-2.9
Diamond	19	16	white, deeply etched	-4.5
Diamond	11	10	white, with deformation lamellae	-4.4
Diamond	1	17	pale yellow, flawless	-3.8
Diamond	4	23	pale yellow, flawless	-4.8

Carbon extraction technique: Samples were heated at 1,000 °C for 45 min in circulating O_2 at 1 atm. The combustion tube contained Pt and Cu oxides to oxidize any CO produced during the experiment. 97% of the SiC combusted during the first 5 min, and the remainder combusted within the next 15 min. Yields were better than 99%.

* $\delta^{13}\text{C} = [({}^{13}\text{C}/{}^{12}\text{C})_{\text{sample}}/({}^{13}\text{C}/{}^{12}\text{C})_{\text{standard}}] - 1$, where the standard was Pee Dee belemnite (PDB).

result of an incomplete displacive transformation, or a quenched high-quartz structure. Crystallization of SiO_2 as quartz instead of coesite indicates a maximum pressure of <20–28 kbar at 1,000–1,200 °C, estimated using equilibrium lines of high quartz/low quartz¹⁷ and quartz/coesite¹⁸ transitions. Under the above pressure and temperature conditions, the stable polymorph of SiO_2 is high quartz, and not tridymite or cristobalite which are stable only at pressures below 10 kbar¹⁹. Thus, β -SiC must have crystallized in a more oxidizing environment at a depth of 60–90 km where the temperature may be ~1,000 °C, the minimum temperature at which SiC can be synthesized in the laboratory¹⁶.

Results of our carbon isotope analyses are presented in Table 1. The $\delta^{13}\text{C}$ values of diamond samples fall in the range, -2.9‰ to -4.8‰ (average = -4.1‰), whereas the value for α -SiC is -24.0‰, indicating a strong ^{12}C enrichment in SiC, ~20%. Large differences in isotopic compositions have also been observed in diamonds from worldwide localities^{20–22}. Hypotheses proposed to account for such isotope heterogeneities include multiple sources of mantle carbon²³, introduction of organic carbon by subduction²⁴, and extreme fractionation by degassing of the mantle²⁵, all suggestive of disequilibrium growth of diamonds. As no evidence from diamond research is presently available to justify these hypotheses, we shall discuss an alternative.

Isotopically zoned but non-coated diamonds^{23,26} show variations in ^{13}C from core to rim of only about 4‰. We suggest that the 20‰ fractionation observed between SiC and diamond is due to a distillation process: preferential uptake of ^{13}C during the growth of SiC enriched the carbon reservoir, in which the diamond later grew, in ^{12}C . Evidence that the SiC was formed first is provided by the presence of SiC inclusions in diamonds from Fuxian¹³. To explain the preferential uptake of ^{13}C by SiC, we examined the thermodynamic properties (1 atm) of diamond²⁷ and 6H SiC²⁸ and found that SiC has much higher enthalpy and entropy than diamond. Deines²⁹ noted that in an isotope exchange reaction, the solid of lower heat capacity can be correlated with higher vibrational frequency and a stronger tendency to incorporate the heavy isotope. This suggests that SiC should be enriched in ^{13}C and diamond in ^{12}C , as we observe.

In studies of iron meteorites^{30,31}, systematic ^{12}C enrichment in graphite and ^{12}C depletion in cohenite (iron carbide), in 12‰ fractionation at ~600 °C was observed, and the fractionation was expected to increase with increasing temperature. The ^{13}C - ^{12}C systematics observed in our SiC-diamond system is completely analogous. Our isotope data imply that the carbon reservoir in the mantle beneath Fuxian has a $\delta^{13}\text{C}$ value between -24‰ and -4‰, and is probably much lower than -5‰, a value derived from isotope analysis of natural diamonds. A more precise estimate would require a knowledge of all fractionation

processes and the number of carbon-bearing phases participating in the isotope exchange reaction. □

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Rb–Sr dating of sphalerites from Tennessee and the genesis of Mississippi Valley type ore deposits

Shun'ichi Nakai, Alex N. Halliday,
Stephen E. Kesler & Henry D. Jones

Department of Geological Sciences, University of Michigan, Ann Arbor,
Michigan 48109-1063, USA

MISSISSIPPI Valley type (MVT) ore deposits commonly consist of some combination of lead, zinc and iron sulphides accompanied by barite, fluorite, dolomite and calcite. They are thought to form by fluid expulsion from sedimentary successions^{1–3}, but their exact origin has remained controversial because of the scarcity of reliable geochronological data. In a recent and widely quoted model the MVT deposits of the central and eastern United States formed as a result of large-scale lateral fluid flow driven by the tectonic squeezing of sedimentary successions during the late Palaeozoic Alleghenian orogeny (330–250 Myr)⁴. Here we report the first direct Rb–Sr dating of sphalerites from an MVT deposit and propose this as a useful geochronological technique for sulphide mineralization with poor age control. Rb–Sr data for sphalerites from Coy mine, East Tennessee, define an isochron age of 377 ± 29 Myr, ruling out any genetic connection with the Alleghenian orogeny. Instead, MVT mineralization in the eastern United States was apparently generated from fluids expelled during thrusting associated with the older Acadian orogeny (380–350 Myr).

The difficulty in isotope dating the MVT deposits is due to

TABLE 1 Rb–Sr data for sphalerites and their fluid inclusions

Sample number		Rb (p.p.m.)	Sr (p.p.m.)	$^{87}\text{Rb}/^{86}\text{Sr}$	$^{87}\text{Sr}/^{86}\text{Sr}$	Apparent sphalerite-fluid age (Myr)*
C5L	r	0.06686	0.2805	0.6868	0.714635 (69)†	358 ± 21
	l	63.05	3.393	0.05349	0.711404 (85)	
C14I	r	0.2943	1.462	0.5796	0.713633 (19)	306 ± 8
	l	142.1	5.539	0.07387	0.711428 (13)	
C14II	r	0.3364	0.8808	1.101	0.716706 (22)	373 ± 8
	l	150.4	9.984	0.04337	0.711096 (47)	
C15	r	0.2290	1.056	0.6247	0.712408 (20)	150 ± 9
	l	371.1	979.8	0.1090	0.711308 (37)	
C16D	r	0.1707	1.386	0.3549	0.712483 (16)	333 ± 11
	l	47.16	2.403	0.05652	0.711070 (18)	
C16L	r	0.4755	1.858	0.7374	0.714931 (20)	441 ± 9
	l	73.58	1.697	0.1240	0.711079 (21)	
C18	r	0.05042	0.6601	0.2200	0.711761 (16)	233 ± 24
	l	62.10	2.085	0.08579	0.711316 (50)	
C44	r	0.2631	0.4134	1.835	0.720396 (23)	

Rb and Sr were separated using standard ion-exchange procedures²⁶, and their concentrations measured by isotope dilution using a mixed $^{87}\text{Rb}/^{84}\text{Sr}$ spike. In the first column, r and l denote residue and leachate, respectively. Rb and Sr concentrations of fluid inclusions were calculated assuming that the weight fraction of fluid inclusions in the sphalerites was 300 p.p.m. (see text). Isotope measurements were performed using a VG Sector mass spectrometer. During this study, an average $^{87}\text{Sr}/^{86}\text{Sr}$ value of NBS 987 Sr standard was 0.710256 (5) (where the number in parentheses indicates the 2σ uncertainty). The decay constant λ $^{87}\text{Rb} = 1.42 \times 10^{-11} \text{ yr}^{-1}$ was used for the age calculation.

* Uncertainty calculated by combining run precision on Sr isotopic composition with an uncertainty of 1% (2σ) on the $^{87}\text{Rb}/^{86}\text{Sr}$ ratio.

† 2σ uncertainty.

the lack of suitable minerals with high or variable parent/daughter ratios. This has led to indirect attempts to determine their age. One approach has been to date apparent resetting from hydrothermal fluids. For example, the Rb–Sr age of glauconites has been used to estimate the age of mineralization in the Viburnum Trend⁵ (a 75-km-long belt of MVT mineralization in Missouri), based on the assumption that the formation of the MVT deposit was the last process to affect the isotope system in the glauconites. A second approach is to use model ages. For example, Halliday *et al.*⁶ used the large fractionation in Sm/Nd ratios in fluorites from the North Pennine Orefield to place constraints on the timing of mineralization. Similarly, Kesler *et al.*⁷ estimated that the mineralization in the Mascot–Jefferson City district in East Tennessee was younger than 395 Myr using a crude basin-evolution model. As with model lead ages, the interpretations are critically dependent on the inferred isotope evolution of the source of the components, which is seldom well defined. There have been very few attempts to date ore constituents directly—one notable exception was that of Lange *et al.*⁸ who attempted to date galenas from the Viburnum mine in southeast Missouri using the Rb–Sr method. In addition, Med-

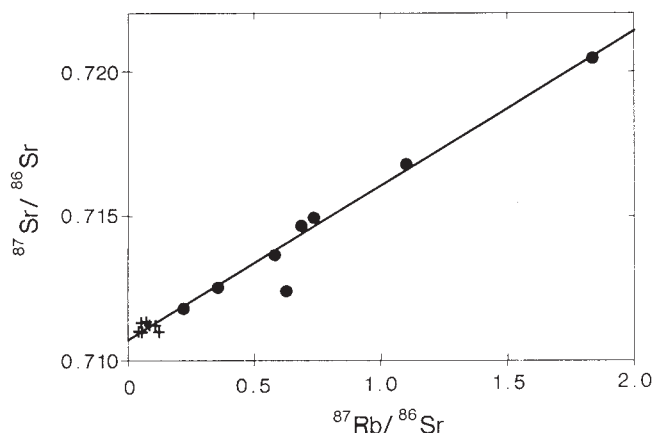


FIG. 1 Rb–Sr isochron of sphalerites from Coy mine. Circles and crosses denote residues and leachates, respectively. Eliminating a point for C15 (see text), the isochron yields an age of 377 ± 29 Myr (mean square of weighted deviates is 62.6).

ford *et al.*⁹ reported Sr isotope data for sulphide and carbonate minerals from Pine Point mine, Canada, and noted that sphalerite can have a high Rb/Sr ratio which could facilitate its use in isotope dating.

The deposits in the Mascot–Jefferson City district, East Tennessee, are hosted by the Lower Ordovician Knox group. The top of the Knox group is marked by an extensive Middle Ordovician unconformity, and the ores below occur in collapse breccias formed by karstification¹⁰. Palaeomagnetic measurements¹¹ and K–Ar age determinations on clays and K-feldspars in this district^{12,13} have been interpreted as indicating large-scale fluid migration in the late Palaeozoic, which led to the view that the Appalachian MVT deposits in East Tennessee were formed by massive movements of fluid expelled from sedimentary basins during the Alleghenian orogeny (330–250 Myr)⁴.

Rb–Sr data for eight leached samples of sphalerite, together with seven of their (fluid inclusion) leachates are presented in Table 1 and Fig. 1. For each analysis ~100 mg of hand-picked, washed sphalerite was crushed in deionized water and leached with the water to remove the fluid-inclusion contents. The absolute concentrations of Rb and Sr in the fluid inclusions can only be estimated because of the inability to measure accurately the mass of the inclusions. Haynes *et al.*¹⁴ have reported that gas chromatographic analyses of fluid inclusions in sphalerites from Mascot–Jefferson City district yielded between 3 and 49 μmol per g H_2O (average 13.5 μmol per g) and that the salinities of the fluid inclusions were ~20%. From these data, we estimate that the weight fraction of fluid inclusions in the sphalerites averaged ~300 p.p.m. Rb and Sr concentrations in the fluid inclusions shown in Table 1 were calculated using this value, and are estimated to be accurate to within an order of magnitude. The Rb/Sr ratios, however, are accurate to better than 1%. After removal of the fluid inclusions, the sphalerites were dissolved in hot 6M hydrochloric and concentrated nitric acid (9:1). The sphalerites have low Sr concentrations (<2 p.p.m.), but variable $^{87}\text{Rb}/^{86}\text{Sr}$ ratios (0.3–2.0).

The isotope data are plotted on a conventional isochron diagram in Fig. 1 and, with the exception of C15, all the sphalerites show a clear correlation between $^{87}\text{Rb}/^{86}\text{Sr}$ ratios and $^{87}\text{Sr}/^{86}\text{Sr}$ ratios. The scatter observed probably reflects heterogeneity in the initial Sr isotopic compositions of the sphalerites—such isotope variability for mineralizing solutions is well documented for MVT deposits^{8,15}. In addition, the introduction of secondary fluids and the deformation of the ore

constituents may also have disturbed the Rb-Sr system. Sample C15 was notably more deformed than the other samples. Ignoring this point, the data for the sphalerites define an apparent isochron age of 377 ± 29 Myr (2σ) with an initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of $0.7107(3)$ (2σ uncertainty).

The leachates (fluid inclusions) have lower $^{87}\text{Rb}/^{86}\text{Sr}$ ratios (<0.1) than the sphalerites and cluster around the lower end of the isochron in Fig. 1. Calculated apparent Rb-Sr ages for leach-sphalerite pairs are shown in Table 1. The ages obtained are extremely variable, ranging from 150 to 440 Myr. These data indicate that in the relatively large samples required for this study, several types of inclusion fluids (known to exist) were sampled, including secondary fluids not directly related to sphalerite precipitation.

Figure 2 shows a chondrite-normalized rare-earth element (REE) pattern for sample C16D (after removal of the inclusion fluids). As far as we are aware, high-precision REE analyses of sphalerites have not been previously reported. Although the concentrations are low, the pattern is strikingly similar to that of average shales¹⁶. If the REEs in the sphalerite were derived from the hydrothermal fluids no major fractionation can have occurred during mineralization.

The exact location of the Rb, Sr and REEs in the sphalerites is uncertain. The Rb/Sr ratio is much too high for the components to be derived from carbonate, fluorite or barite inclusions. From the Rb/Sr ratio (0.12) and Sr/Nd ratio (90) of C16D, clay or feldspar are more likely host phases. Scanning electron microscopy (SEM) of the hand-picked sphalerites did not reveal any silicate phases on the micrometre scale except quartz and very rare feldspar on the surface of some of the grains. The K-feldspar seems to predate sphalerite in all samples that we have examined. The sphalerite and feldspar are distributed together in silica-rich alteration zones¹³ which are thought to be coeval with mineralization¹⁷. Our SEM measurements indicate that feldspar inclusions probably constitute $<0.1\%$ of the hand-picked sphalerite, but at least 0.1% is required to explain the Rb and Sr concentrations of C16D. If feldspar is the host of the REEs, the lack of fractionation of Eu relative to a typical crustal pattern (Fig. 2) would require that it grew under non-reducing conditions. We conclude that it is highly unlikely that the Rb-Sr data reflect a dolomite, barite, fluorite or clay component. The Rb, Sr and REEs are most likely hosted in the sphalerite itself or in K-feldspar. These phases are probably cogenetic, therefore the Rb-Sr data should define the age of sphalerite mineralization. If the Rb, Sr and REEs are not

held in inclusions, but were sequestered from hydrothermal fluids in minute amounts by the sphalerite, it is not clear how the range in Rb/Sr could have been generated. One possibility is that the composition of the ore fluid evolved with deposition of gangue minerals as a result of different bulk partition coefficients for Rb and Sr. Another possibility is that the variations reflect incomplete mixing of fluids. Both of these seem unlikely because the range of Rb/Sr in the fluid-inclusion contents analysed is relatively restricted (Table 1).

The geologically reasonable isochron age implies that the range in initial Sr isotopic composition in the ore fluids must have been relatively small. Kesler *et al.*⁷ and Kessen *et al.*¹⁸ reported Sr isotope data for MVT deposits in East Tennessee and showed that the gangue minerals that precipitated from the early dolomite-forming fluids had similar Sr isotopic composition to the host carbonates ($^{87}\text{Sr}/^{86}\text{Sr} = 0.7090\text{--}0.7092$)⁷ whereas $^{87}\text{Sr}/^{86}\text{Sr}$ ratios for later gangue minerals increased with time up to 0.7122 for paragenetically late calcite. The initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio obtained for sphalerites from the isochron intercept in this study (0.7107) is consistent with this trend, being intermediate between the Sr isotopic ratio for pre-ore and post-ore dolomites. Therefore the isotope data are consistent with the idea that the Sr in the sphalerite was sequestered from the ore fluids themselves rather than inherited from unrelated older material in the ore. Furthermore the combined data indicate a relatively small range in Sr isotopic composition of the ore-forming fluids at the time of mineralization.

Our data indicate that mineralization predated the Alleghenian orogeny (330–250 Myr) by ~ 50 Myr and are therefore inconsistent with the hypothesis that basinal brines tectonically expelled during that event played an important part in ore genesis. Furthermore the alkali feldspar intergrown with sphalerite in these deposits cannot have grown during Alleghenian fluid movements as argued by Hearn *et al.*¹⁵. Although it is generally considered that the thermal effects of the Acadian orogeny (380–350 Myr) were restricted to areas further east than this district¹⁹, it has been shown that there was major thrusting immediately to the southeast of the district at this time²⁰. It seems therefore that uplift and deformation in early Devonian resulted in fluid expulsion and the formation of MVT mineralization to the west. MVT deposits located along the Appalachian mountains from Georgia to Newfoundland have many features in common, such as high Zn/(Zn+Pb) ratios and relatively homogeneous Pb and stable isotopic compositions. Among the Appalachian MVT deposits, the mineralization in the St George Group in Newfoundland is similar in stratigraphic setting to that in East Tennessee¹⁰. ^{40}Ar – ^{39}Ar ages of alkali feldspar from MVT ore deposits in western Newfoundland were reported by Hall *et al.*²¹. They concluded that the alkali feldspars were subjected to thermal events at 370–350 Myr and 210 Myr and interpreted the older event as the time of sphalerite mineralization²¹. These data are consistent with the suggestion that Appalachian MVT mineralization was related on a regional scale to the Acadian orogeny.

Oilfield brines are often considered to be suitable ore-forming solutions for MVT deposits²². They can have high $^{87}\text{Sr}/^{86}\text{Sr}$ ratios owing to interactions with silicate minerals in sedimentary successions. The ratios for several oilfield brines range between 0.709 and 0.735^{23,24} and our initial Sr isotopic ratio for the sphalerites and the fluid inclusions falls in this range. Furthermore, Chaudhuri *et al.*²⁵ have reported Rb/Sr ratios for oilfield brines from Kansas of ~ 0.02 , similar to the ratios found in the fluid inclusions from East Tennessee. Our data are therefore consistent with basin-brine-expulsion models for the fluids that formed both the MVT deposit and the fluid inclusions. □

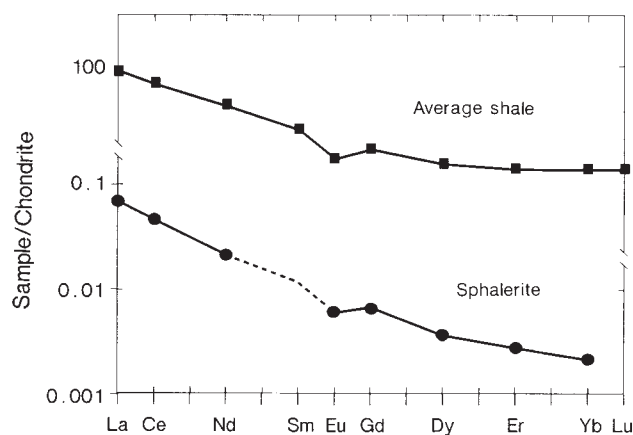


FIG. 2 Chondrite-normalized pattern of a sphalerite, C16D and North American Shale Composite (NASC). The REE abundances in the Leedey chondrite²⁷ are used to normalize the values. The point for Sm was estimated by interpolating points for Nd and Gd. The pattern of the sphalerite is similar to that of typical crustal material, NASC.

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Experimentally induced life-history evolution in a natural population

David A. Reznick*, Heather Bryga* & John A. Endler†

* Department of Biology, University of California, Riverside, California 92521.

† Department of Biological Sciences, University of California, Santa Barbara, California 93106, USA

LIFE-HISTORY theory predicts that reduced adult survival will select for earlier maturation and increased reproductive effort; conversely, reduced juvenile survival will select the opposite^{1–5}. This is supported by laboratory studies^{6–10} and comparative data from natural populations^{11–15}. Laboratory studies may support a theory, but cannot assess its importance in natural populations, and comparative studies reveal correlations, not causation¹⁶. Long-term perturbation experiments on natural populations resolve both problems. Here we report the findings of a long-term study of guppies (*Poecilia reticulata*), in which the predictions of life-history theory are supported. Life-history differences among populations of guppies are closely associated with predator species with which guppies live^{13,17–21}. The predators apparently alter age-specific survival because they are size-specific in their choice of prey^{21–23}. *Crenicichla alta* (a cichlid), the main predator at one class of localities, preys predominantly on large, sexually mature size classes of guppies^{22–24}. *Rivulus hartii* (a killifish), the main predator at another class of localities, preys predominantly on small, immature size classes. Guppies from localities with *Crenicichla* mature at an earlier age, have higher reproductive effort, and have more and smaller offspring per brood than those from localities with just *Rivulus*. These differences are heritable, and correspond with theoretical predictions^{17–19}. To prove that predation caused this pattern, we perturbed a natural population of guppies by changing predation against adults to predation against juveniles. This resulted in significant life-history evolution in the predicted direction after 11 years, or 30–60 generations.

In 1976, guppies were transplanted from a site on the Aripo River (Trinidad) with *C. alta* (control site) to a tributary of the Aripo which previously contained *R. hartii* but no guppies (introduction site)²⁴. This manipulation released guppies from selective predation on adults and exposed them to selective predation on juveniles. This treatment should favour guppies

with delayed maturity and decreased reproductive effort, compared to the control site. The founding population size was 200 adults²⁴, consisting of equal proportions of males and non-virgin females. As guppies have sperm storage and multiple mating, the effective founding population size was almost certainly greater than 200; founder effects are therefore unlikely. In all

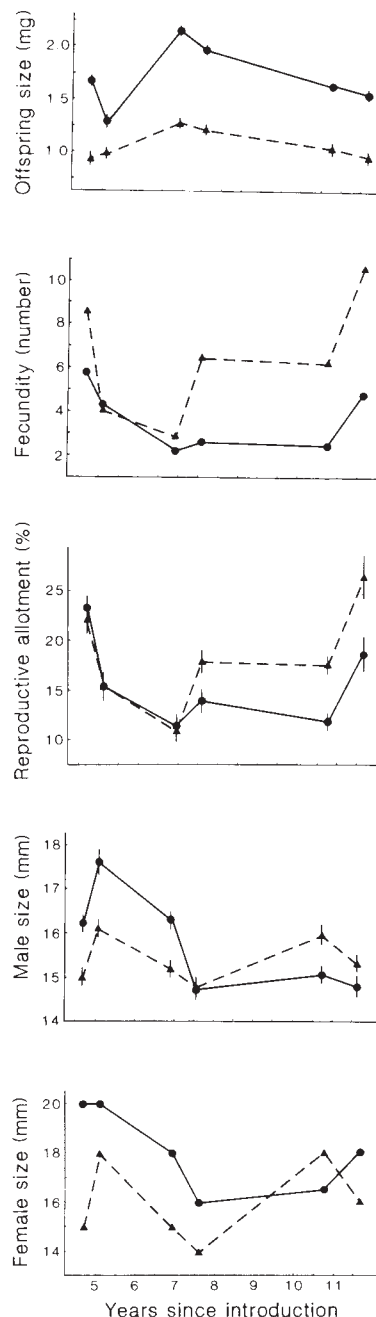


FIG. 1 Life-history phenotypes of guppies from the introduction and control sites. These data are based on wild-caught, field preserved fish. Offspring Size, mean dry weight of developing embryos, corrected for their stage of development and female size. Fecundity, expected number of offspring for a 30 mg (somatic dry weight) female. Reproductive Allotment, per cent of total dry weight that consists of developing embryos, and is corrected for the stage of development of the embryos. Male Size, average size of sexually mature males. Female Size, minimum mm size class in which the majority of females were carrying developing embryos. ●, Introduction site. ▲, Downstream control. All values are least-square means and one standard error from analyses of variance. All statistical comparisons were made within a collection and were executed with the SAS GLM procedure³¹.

TABLE 1 Aripo tributary introduction

Life history trait*	Reznick and Bryga (1987) ²¹		This study		Reznick (1982) ¹⁷	
	Control (<i>Crenicichla</i>)	Introduction (<i>Rivulus</i>)	Control (<i>Crenicichla</i>)	Introduction (<i>Rivulus</i>)	<i>Crenicichla</i> ¹⁷	<i>Rivulus</i> ¹⁷
Male age at maturity (days)	60.6 (1.8)	72.7 (1.8)†	48.5 (1.2)	58.2 (1.4)†	51.8 (1.1)	58.8 (1.0)†
Male size at maturity (mg-wet)	56.0 (1.4)	62.4 (1.5)†	67.5 (1.2)	76.1 (1.9)†	87.7 (2.8)	99.7 (2.5)†
Female age at first parturition (days)	94.1 (1.8)	95.5 (1.8) (NS)	85.7 (2.2)	92.3 (2.6)‡	71.5 (2.0)	81.9 (1.9)†
Female size at first parturition (mg-wet)	116.5 (3.7)	118.9 (3.7) (NS)	161.5 (6.4)	185.6 (7.5)†	218.0 (8.4)	270.0 (8.2)†
Brood size, litter 1	2.5 (0.2)	3.0 (0.2) (NS)	4.5 (0.4)	3.3 (0.4)‡	5.2 (0.4)	3.2 (0.5)†
Brood size, litter 2	6.3 (0.3)	7.0 (0.3)§	8.1 (0.6)	7.5 (0.7) (NS)	10.9 (0.6)	10.2 (0.8) (NS)
Brood size, litter 3	—	—	11.4 (0.8)	11.5 (0.9) (NS)	16.1 (0.9)	16.0 (1.1) (NS)
Offspring size (mg-dry), litter 1	0.91 (0.02)	0.87 (0.02) (NS)	0.87 (0.02)	0.95 (0.02)§	0.84 (0.02)	0.99 (0.03)†
Offspring size, litter 2	0.93 (0.02)	0.86 (0.02)‡	0.90 (0.03)	1.02 (0.04)‡	0.95 (0.02)	1.05 (0.03)‡
Offspring size, litter 3	—	—	1.10 (0.03)	1.17 (0.04) (NS)	1.03 (0.03)	1.17 (0.04)†
Interbrood interval (days)	24.9 (0.4)	24.89 (0.4) (NS)	24.5 (0.3)	25.2 (0.3) (NS)	22.8 (0.3)	25.0 (0.03)†
Reproductive effort (%)#	4.0 (0.1)‡	3.9 (0.1) (NS)	22.0 (1.8)	18.5 (2.1) (NS)	25.1 (1.6)	19.2 (1.5)‡

Values are means (s.e.), and represent data from refs 17 and 21, or this study.

NS, not significant.

* Differences in mean values among experiments are attributable to differences in food availability. Ref. 17 had the highest levels, this study was intermediate, and ref. 21 had the lowest levels.

† $P < 0.01$.

‡ $P < 0.05$.

§ $0.05 < P < 0.10$.

|| Fish were only kept until they produced two litters of young in ref. 21.

Values for reproductive effort in ref. 21 represent a single estimate made at the end of the experiment; those for the other two studies represent the sum of four consecutive estimates. See ref. 17 for details on the latter analysis.

later visits to the field site, we found guppy populations to be large and widespread. Thus, subsequent genetic drift also seems unlikely.

Two years after the transfer, the phenotypes of females caught from the introduction site differed from those of females from the control site in offspring size and reproductive allotment (embryo weight/total body weight), suggesting a response to changed predation¹³. Six subsequent samples, collected between March 1981 and March 1988, revealed persistent differences between fish from these localities for many life-history variables (Fig. 1). Females from the introduction site produced larger offspring and initiated reproduction at a larger size than those from the control site. They also had smaller brood sizes and smaller reproductive allotments in the four dry-season samples (in (month/year) 3/81, 2/84, 4/87, 3/88), but not in the two wet-season samples (8/81, 6/83). We previously found, in a large-scale survey of many streams, that there was a similar trend towards lower reproductive allotment, lower fecundity and reduced differences between guppies from *Rivulus* and *Crenicichla* localities during the wet season²⁰. Mature males from the introduction site were significantly larger than control site males prior to 1984, but smaller thereafter. With the excep-

tion of male size, the phenotypes of all variables over 11 years supports the predictions of life-history theory.

To determine whether these differences were heritable, we collected guppies from both localities in March 1987, then reared their descendants through two generations in a common environment, using the methods of our previous studies^{17-19,21}. Rearing fish through two generations in a common environment eliminates environmental influences on the life history; remaining differences are assumed to have a genetic basis. Males and females from the introduction site mature at a significantly later age and larger size than those from the control site (Table 1); they also produce significantly fewer young in the first brood, but show no differences in the size of the second or third broods. This pattern of increased fecundity early in life corresponds to the results of laboratory selection experiments^{10,25} and supports the hypothesis that early-life performance has a stronger influence on fitness than later-life performance²⁴⁻²⁶.

Guppies from the introduction site produce larger offspring in all three broods, although the difference is only significant for the second brood. They have lower reproductive effort at 6, 8 and 10 weeks after the initiation of controlled feeding (Fig. 2), although the difference was only significant at 8 weeks. The differences in reproductive effort early in life are attributable to the delayed age of first parturition and smaller initial brood sizes in the introduction site guppies. All of these patterns match those observed among fish from two *Crenicichla* and two *Rivulus* sites considered in our earlier study¹⁷ (Table 1). It is therefore clear that the introduction site guppies have evolved genetically new life-history parameters that correspond to the usual differences between these two types of localities.

The genetic differences in life histories reported here are far greater than those observed previously in a similar study²¹ (Table 1); although both studies revealed equally strong differences in the life history phenotypes of wild-caught fish. The different results may reflect differences in the duration of the experiments (4 versus 11 years), and may illustrate the time required for a response to selection, arguing strongly for long-term field experiments. As these experiments are duplicates, the general similarity in their results, plus their convergence upon natural populations under the same predation regime, suggest that these are specific responses to natural selection, rather than the results of drift or founder effects.

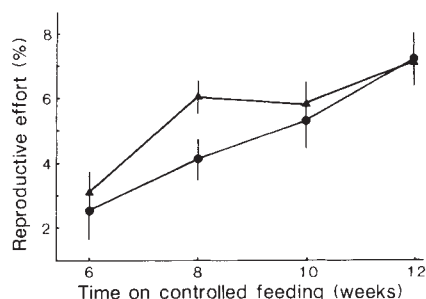


FIG. 2 Reproductive effort in the second generation, laboratory-reared fish at 6, 8, 10 and 12 weeks after the initiation of controlled food availability (following the methods of ref. 19). ▲, Downstream control; ●, Introduction site. Controlled feeding began when the fish were 25 days old. These time intervals correspond to routine changes in food availability. Earlier ages were not included because most females had not yet conceived their first litter. Later ages were not included because many females had already produced their third litter and were no longer in the experiment.

These results demonstrate significant evolutionary changes in the life histories of introduction-site guppies, which are consistent with theoretical predictions. They provide direct experimental field evidence of the importance of predation in moulding life history evolution in guppies, though of course other factors may be important²¹. More generally, these results provide evidence that mean differences in age-specific survival will mould the evolution of life-history patterns. The widespread evidence for size-specific predation in other species²⁷⁻³⁰ suggests that this could be a common factor in life-history evolution. □

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Formation of target-specific neuronal projections in organotypic slice cultures from rat visual cortex

Jürgen Bolz, Ninoslav Novak, Magdalena Götz & Tobias Bonhoeffer*†

Friedrich-Miescher-Labor der Max-Planck-Gesellschaft, Spemannstrasse 37-39, 7400 Tübingen, West Germany

* Max-Planck-Institut für biologische Kybernetik, Spemannstrasse 38, 7400 Tübingen, West Germany

A CHARACTERISTIC feature of the mammalian cortex is that projection neurons located in distinct cortical layers send their axons to different targets. In visual cortex, cells in layers 2 and 3 project to other cortical areas, whereas cells in layers 5 and 6 project to subcortical targets such as the lateral geniculate nucleus^{1,2}. The proper development of these projections is crucial for correct functioning of the visual system. Here we show that specific connections are established in an organotypic culture system in which rat visual cortex slices are co-cultured with another slice of the visual cortex or with a thalamic slice. The laminar

origin and cellular morphology *in vitro* of cortical projections to other cortical regions or to subcortical targets are remarkably similar to those seen *in vivo*. In addition, axons of projecting cells are not restricted to particular pathways, but appear instead to grow directly towards their appropriate target. These observations raise the possibility that chemotropic attraction from the target areas may play an important part in the development of the cortical projection pattern.

Slices of rat visual cortex were co-cultured either with a slice from the lateral thalamus or another slice of visual cortex using a roller-culture technique³. We used animals from postnatal day 0 up to postnatal day 2, an age at which the axons of cortical projection neurons in rat have not yet reached their targets⁴⁻⁶. The slices were placed side by side less than 1 mm apart on glass coverslips and embedded in a plasma clot (Fig. 1a). As reported previously⁷, cultures maintained in this manner flatten to one to three cell layers within about 10 days *in vitro*, contain all major cortical cell types and survive for several weeks. In the experiments described here, co-cultures were examined after 8-14 days *in vitro*.

As we were interested in the laminar origin of cortical projection neurons *in vitro*, we studied the layering of the cultured slices in detail. In the rat, the deepest cortical layer (sublayer 6B) forms a band of tightly packed cells lying immediately above the white matter⁸. This cell band was clearly visible in all Nissl-stained slice cultures and formed a sharp border with the white-matter zone (Fig. 1c). Usually, the remaining cortical layers could also be identified in cultures maintained for 1-2 weeks and were comparable to those in the normal visual cortex from animals of that age (Fig. 1b). The cortical layers of young animals, however, are less distinct than in the adult, and in slice cultures it was sometimes difficult to determine the exact boundaries of every layer. Therefore we used an additional approach to demonstrate the layering in our culture system, taking advantage of the fact that the cortical layers are born in an inside-first outside-last sequence^{9,10}. Cells destined for different layers were labelled on their birth by injecting timed pregnant rats with 5-bromodeoxyuridine (BrdU; see Fig. 2 methods). We then prepared slice cultures from 0-2-day-old animals and compared the laminar distribution of labelled cells after 1-3 weeks *in vitro* with the location of labelled cells in littermates of the corresponding age. *In situ*, most cells generated on embryonic day 16 (E16) are located in the deep cortical layers 5 and 6, but cells generated on E18 are concentrated in the upper layers, 2 and 3 (ref. 11; Fig. 2a and c). In slice cultures, BrdU-labelled cells also formed a distinct band that was only slightly more scattered than *in vivo*. The laminar pattern was very much like the situation *in vivo* at the corresponding age (Fig. 1d and Fig. 2). Thus cortical cells in slice cultures are located at their appropriate laminar position.

The origin and cellular morphology of cortical neurons projecting to thalamic slices *in vitro* were determined by placing a small crystal of the lipophilic fluorescent dye DiI in the thalamic explant (see Fig. 3 methods). A micrograph and camera lucida drawing of these cells in a cortical culture are shown in Figs 3 and 4a respectively. All but one of the labelled cells were located in the deep cortical layers, precisely where cells projecting to the thalamus are found *in vivo*. In these experiments the thalamus was placed immediately adjacent to the white-matter side of the cortical slice. It is possible that only cells in the deep layers of the cortex, that is, those cells closest to the thalamus, are able to project to this target. To rule out this explanation we placed the thalamus next to the pial surface of the cortical culture, far away from the white matter. Again, axonal innervation of the thalamic explant originated from cells in the deep cortical layers. Cells in the superficial layers, although closer in distance to the thalamic explant, did not innervate this target (Fig. 4b). The axons of the deep cortical cells grew directly towards the thalamic explant. In many instances, axon collaterals separate from the main axon at right angles and run horizontally for

† Present address: The Rockefeller University, Laboratory of Neurobiology, 1230 York Avenue, New York, New York 10021, USA.

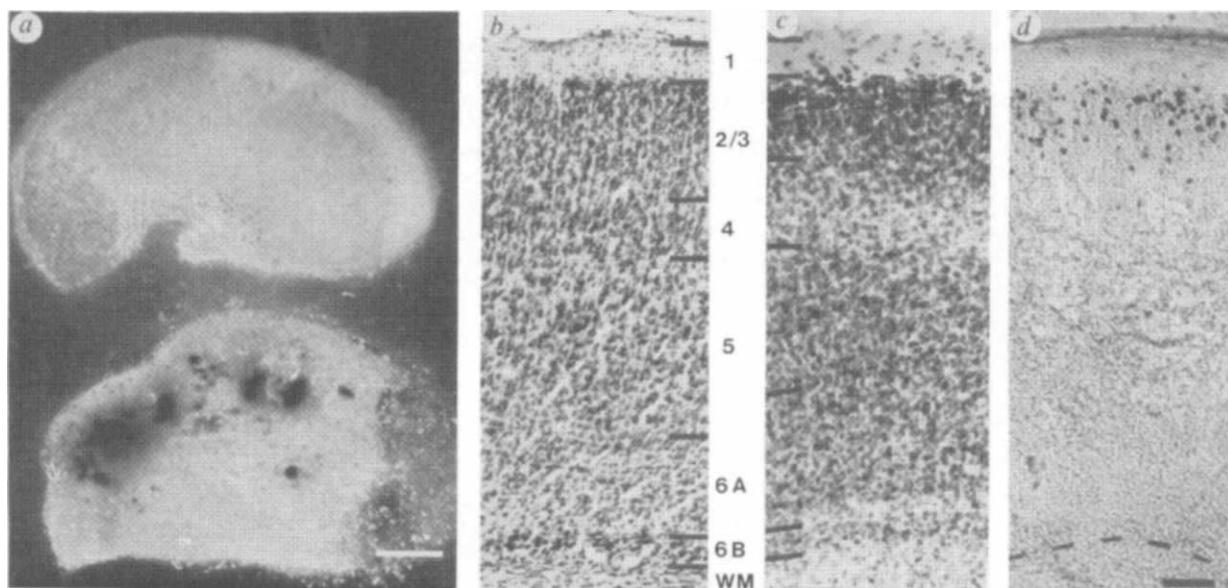


FIG. 1 *a*, Dark-field micrograph of a cortex-thalamus co-culture from a newborn rat fixed after 8 days *in vitro*. The thalamus (below) faces the white-matter side of the cortex (above). The dark spots in the thalamus are DiI crystals that were inserted to trace retrogradely labelled neurons innervating the thalamus *in vitro* (see Fig. 3). Scale bar, 500 μm . *b*, Nissl-stained section from a normal 12-day-old animal. *c*, Nissl-stained cortical slice culture from a new-born animal after 12 days *in vitro*. *d*, BrdU-labelled cells in a slice culture from a 1-day-old animal after 10 days *in vitro*. BrdU was injected on E20 (compare with Fig. 2). Scale bar for *b*, *c* and *d*, 100 μm . WM, white matter.

METHODS. Cortical slice cultures were prepared using the roller-culture technique³ as described previously⁷. In brief, small blocks from the dorsal occipital cortex were cut with a McIlwain tissue chopper at a thickness of 300 μm . Thalamic slices were obtained from a block of tissue containing the dorsal and ventral lateral geniculate nucleus and parts of the surrounding

lateral posterior nucleus. After cutting, the slices were stored for 30–45 min at 4 °C in Geys balanced salt solution supplemented with glucose. A thalamic slice and a cortical slice were then placed adjacent to each other on a glass coverslip. In some co-cultures the thalamus was placed close to the white matter, and in others close to the pial side of the cortical slice. The tissue sections were then embedded in chicken plasma coagulated with thrombin. The slices were kept in plastic culture tubes (Nunc) and maintained with a medium consisting of 50% Eagle basal medium, 25% Hank's balanced salt solution and 25% horse serum containing 0.1 mM glutamine and 6.5 mg ml⁻¹ glucose (all from Gibco). The culture tubes were put in a roller drum incubator at 36 °C. After 3 days in culture, mitotic inhibitors (5-fluoro-2-deoxyuridine, cytosine-beta-D-arabino-furanoside and uridine; concentration 10 μM) were added to the medium for 24 h to prevent excessive growth of glial cells and fibroblasts.

some distance within the white-matter or in the deep cortical layers. After travelling for up to 1.5 mm, axons then make a second sharp turn and grow through the grey matter to head straight for the thalamus. In control experiments ($n=24$ cultures) small crystals of DiI were placed near the pial side of the cortical cultures but away from the co-cultured thalamus. When examined after 3–15 days *in vitro*, all cells labelled either through their axon or through their apical dendrites were confined to a restricted region in the culture next to the DiI crystal (distance <500 μm). Therefore, it seems unlikely that

corticothalamic fibres have found their target by initial random outgrowth, sending axonal branches in all possible directions and then eliminating all the branches but the one that hit the target. Instead, the axons of the deep cortical cells probably grow directly towards the thalamic explant.

In both sets of experiments, most of the cells innervating the co-cultured thalamus were located in the deep cortical layers (40 out of 44 cells in 11 co-cultures). Cells in the superficial layers do not innervate this target. One possible explanation for this is that superficial layer cells are not capable of establishing

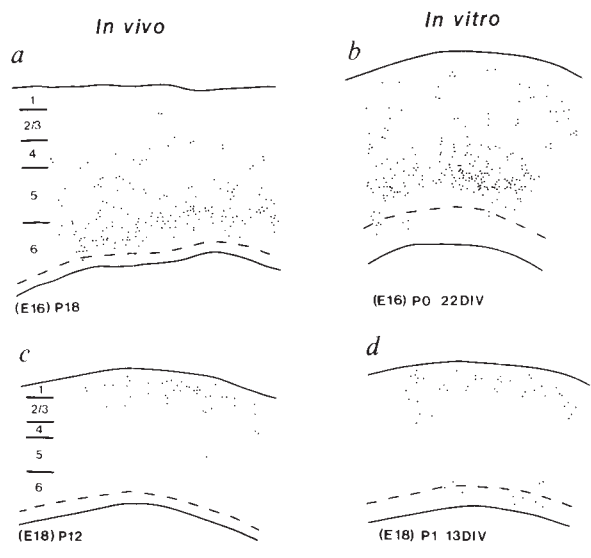


FIG. 2 Distribution of labelled cells in visual cortex *in vivo* and in cortical slice cultures resulting from an injection of BrdU on different embryonic days. The cortical layers *in vivo* (numbers on the left) and the borders between the grey matter and white matter (dashed lines) were determined after counterstaining with cresyl violet. Cell counts were made from larger regions as displayed here. *a*, Labelled cells in an 18-day-old animal after BrdU injection on E16; 87% ($n=254$) of the cells are in the lower half of the grey matter. *b*, BrdU-labelled cells in a slice culture prepared from a littermate at the day of birth and kept *in vitro* for 22 days; 71% ($n=572$) of the cells are in the lower half of the grey matter. *c*, BrdU-labelled cells in a 12-day-old animal after injection on E18; 93% ($n=115$) of the cells are in the upper quarter of the grey matter. *d*, BrdU-labelled cells in a culture prepared from a 1-day-old littermate after 13 days *in vitro*; 89% ($n=380$) of the cells are in the upper quarter of the grey matter.

METHODS. Timed, pregnant rats were injected on different embryonic days with 200 mg per kg BrdU (Serva, UK). Cells that are generated at the time of injection incorporate BrdU (ref. 35); they were detected with immunohistochemical methods and drawn from the microscope with the aid of a camera lucida.

projections *in vitro*. Alternatively, these cells may not recognize the thalamus as an appropriate target, as, *in vivo*, cells in the upper layers participate in long-range corticocortical projections and do not innervate the thalamus. To examine these possibilities we prepared cultures from two cortical slices placed next to each other. Figure 4c shows an example of labelled neurons after application of Dil in the co-cultured cortex. Many retrogradely labelled neurons are found in the superficial layers (126 out of 201 cells in 7 co-cultures), the remaining cells are in the lower layers. The laminar distribution pattern of the labelled cells is very similar to the distribution of corticocortical cells *in vivo*, being mainly situated in the upper layers, but some are also found in layer 5 and in upper layer 6 (refs 12–15).

Not only do the cortical projections established *in vitro* originate specifically from the appropriate layers, but they also seem to be formed by the correct cell type within each layer. First, all projecting neurons have the characteristic morphology of pyramidal cells. Even though several types of intrinsic neurons are known to be present in cortical cultures^{7,16}, we never observed such neurons with long axonal projections into either the thalamic or the cortical slices. Moreover, there is a close relationship between the pyramidal cell morphology and the efferent target *in vivo*. For example, pyramidal cells in layer 6 that project to the dorsal lateral geniculate nucleus (dLGN) have apical dendrites never reaching higher than layer 3 (ref. 17). Subcortical targets of layer 5 cells include the ventral LGN (vLGN), the lateral posterior (LP) nucleus and the superior colliculus^{12,18–20}. *In vivo*, subcortically projecting cells examined so far in layer 5 possess a prominent apical dendrite that always forms a large tuft in layer 1^{12,19,20}. They are strikingly different from corticocortically projecting cells in layer 5, whose apical dendrite is short and less branched¹². Projection neurons *in vitro* exhibit the same variations in dendritic morphology. In the cortical slices co-cultured with the dLGN, vLGN and parts of the LP nucleus (see Fig. 1 methods), projection neurons situated close to the white matter in layer 6 have short apical dendrites. In contrast, many cells in layer 5 in cortex–thalamus co-cultures

have a long apical dendrite that extends up towards the pial surface where it often forms an apical tuft. But projection neurons in cortex–cortex co-cultures located in the same laminar position have short apical dendrites.

Since the pioneering studies of Cajal²¹ and later those of Sperry²², several mechanisms have been suggested to explain how specific connections might develop in the vertebrate nervous system. For example, it has been proposed that growing axons are guided by mechanical channels in the neuroepithelium²³, by local chemical cues expressed along the pathway²⁴, by diffusible factors from intermediate guideposts²⁵ or directly from the target^{26–28}. Relatively few studies have examined the pathfinding mechanisms responsible for establishing efferent projections from the mammalian cerebral cortex. Recently it was proposed that the connections from the cortex to the subcortical targets are laid down by the earliest neurons generated in the cortex, the subplate cells, which act as pioneers for corticofugal axons²⁹. There is some controversy as to whether

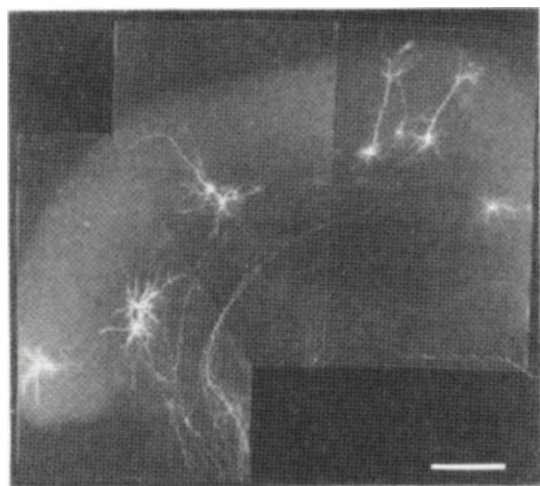


FIG. 3 Fluorescent micrograph of projection neurons in a cortical slice culture retrogradely labelled from the co-cultured thalamus. The pial side is up, the thalamic explant (not shown in the micrograph) faces the white matter zone of the cortical culture. A drawing of these cells is shown in Fig. 4a. Scale bar, 500 μ m.

METHODS. Cortex–thalamus co-cultures were cultivated for 8–14 days as described in Fig. 1, then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. To visualize the projection neurons, small crystals of Dil (Molecular Probes) were placed in the thalamus³⁶. After the co-cultures had been stored in fixative for 6–8 weeks to allow diffusion of the dye within the neuronal membranes, retrogradely labelled cells were viewed with a fluorescent microscope and drawn with the aid of a camera lucida using a $\times 25$ objective. The trajectories of the axons within the thalamus could not be traced as they were masked by the bright fluorescence of the Dil crystals.

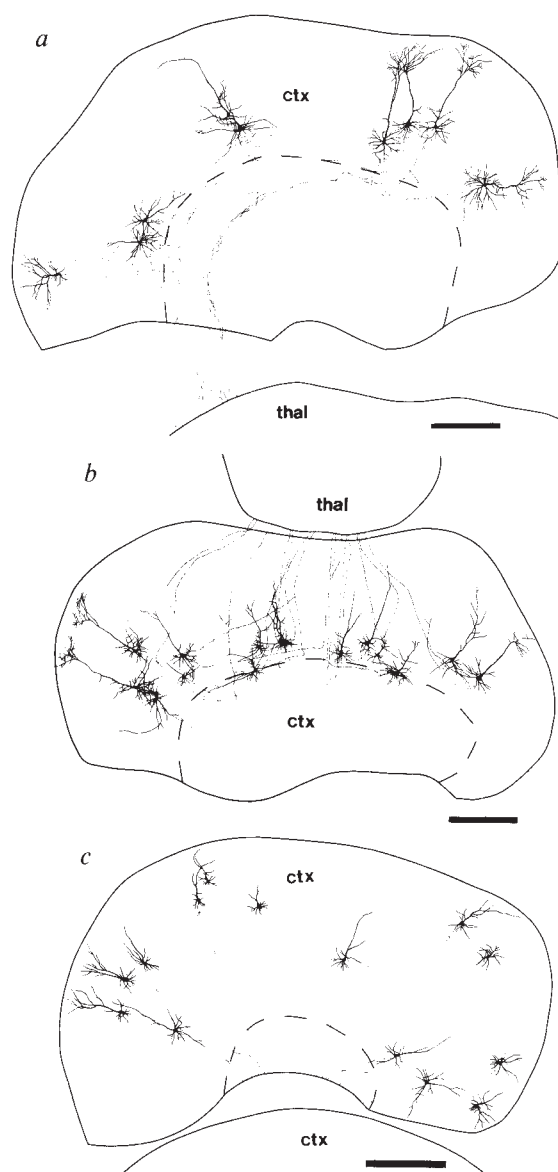


FIG. 4 Camera lucida drawings of cortical projection neurons *in vitro*, retrogradely labelled with Dil from co-cultured thalamus (thal) or cortex (ctx). The thalamus is placed towards the white matter (a) or close to the pial surface of the cortical slice (b). c, Projection neurons in a cortex–cortex co-culture. The borders between the grey and white matter (dashed lines) were determined after the cultures were stained with cresyl violet. Scale bars, 500 μ m.

subplate cells are transitory cells that disappear after birth^{30,31} or whether they remain present throughout the animal's life⁸. Thus, it is not clear whether these cells are present at all in our cultures taken from postnatal animals. Our retrogradely stained projection neurons did not, however, show the characteristic morphological features of subplate cells^{8,32,33}. It thus seems that the projection of subplate cells is not necessary for the establishment of cortical projections *in vitro*. In some, but not all co-cultures, we also observed ingrowing thalamic afferents into the cortical cultures (N.N., V. Staiger, T.B. and J.B., manuscript in preparation). But in all co-cultures shown in Fig. 4, no thalamic afferents were detected despite the intense labelling of many corticofugal cells. It is therefore unlikely that the cortical projections we observed are guided by, or dependent on, ingrowing afferents.

Recent experiments *in vitro* have shown that thalamic explants placed close to the white-matter of cortical explants are innervated by cortical cells from the deep cortical layers³⁴. These findings are confirmed and extended by our data, showing that the laminar position and cellular morphology of cells giving rise to cortical connections established *in vitro* are specific and target-dependent. Furthermore, the present study demonstrates that ectopically positioned projection sites are innervated only by a specific subset of cortical projection neurons, even if their axons have to travel for long distances. The axonal trajectories were always oriented towards the target and not restricted to particular pathways. This suggests that some form of chemotropic attraction may play a part in the formation of specific connections between cortical neurons and their targets. □

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Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport

Stephen C. Hyde, Paul Emsley*, Michael J. Hartshorn*, Michael M. Mimmack, Uzi Gileadi, Stephen R. Pearce†, Maurice P. Gallagher†, Deborah R. Gill, Roderick E. Hubbard* & Christopher F. Higgins‡

Imperial Cancer Research Fund Laboratories,
Institute of Molecular Medicine, University of Oxford,
John Radcliffe Hospital, Oxford OX3 9DU, UK

* Department of Chemistry, University of York, Heslington,
York YO1 5DD, UK

THE ATP-binding cassette (ABC) superfamily of transport systems now includes over thirty proteins that share extensive sequence similarity and domain organization (reviewed in refs 1–3). This superfamily includes the well characterized periplasmic binding protein-dependent uptake systems of prokaryotes, bacterial exporters, and eukaryotic proteins including the P-glycoprotein associated with multidrug resistance in tumours (MDR), the STE6 gene product that mediates export of yeast α -factor mating pheromone, pMDR that is implicated in chloroquine resistance of the malarial parasite, and the product of the cystic fibrosis gene (CFTR). Here we present a tertiary structure model of the ATP-binding cassettes characteristic of this class of transport system, based on similarities between the predicted secondary structures of members of this family and the previously determined structure of adenylate kinase. This model has implications for both the molecular basis

of transport and cystic fibrosis and provides a framework for further experimentation.

Transporters in this superfamily of transport proteins each seem to be relatively specific for a single substrate (or group of related substrates) which can be a small molecule such as an amino acid or sugar, or a large molecule such as a polysaccharide or protein. In general, each transporter consists of four distinct domains that may be fused in a variety of ways (Fig. 1). The ATP-binding domains, which provide the principal distinguishing feature of this family of transport proteins, share about 30% sequence identity over a cassette of about 200 amino acids (Fig. 2) and include the two short ATP-binding motifs present in the nucleotide-binding pockets of many proteins (Walker motifs; refs 4, 5). Proteins containing these ATP-binding cassettes (some of which are not associated with transport; for example, UvrA, a DNA repair enzyme) are designated ABC proteins. It is now clear that hydrolysis of ATP by these cassettes provides the energy requirement for substrate accumulation^{5–10}. As there is no evidence for subunit phosphorylation (ref. 11; C.F.H., unpublished data), this probably involves a conformational change that is transmitted to the transmembrane domains. To understand the mechanism of energy coupling, structural data are required and, in the absence of experimental data, we have derived a tertiary structure model of the ATP-binding cassette.

As a first step in modelling, potential secondary structures were predicted from the primary sequences of ABC-proteins using the Chou-Fasman algorithm¹². Confidence in the predicted consensus secondary structures (Fig. 2) was enhanced by the availability of so many closely related proteins. Folding this linear secondary structure map into a tertiary structure must be based on an experimentally determined structure. Comparison of the secondary structure map of the ABC proteins with that of several ATP-binding proteins whose structures have been determined (adenylate kinase (ADK); phosphoglycerate kinase (PGK); phosphofructokinase (PFK); hexokinase (HK), and pyruvate kinase (PK); refs 13–16, and T. N. Bryant, P. J. Shaw, N. P. Walker, P. L. Wendell and H. C. Watson, unpublished results), showed remarkable similarity to that of adenylate kinase

† Present addresses: Department of Biochemistry, University of Dundee, Dundee DD1 4HN, UK (S.R.P.) and Department of Microbiology, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3JG, UK (M.P.G.).

‡ To whom correspondence should be addressed.

(Fig. 2). The most important difference was an additional loop predicted for the ABC proteins (loop 2) inserted between α -helices C and E of ADK. Assuming the ABC proteins fold in a similar manner to ADK¹³, this loop would be inserted at the surface of the protein and would not be expected to perturb the overall structure. Insertion or deletion within loops does not necessarily perturb the structure of a nucleotide-binding fold¹⁷.

A tertiary structure of the ABC cassette was built in two stages using the protein modelling facilities within QUANTA and CHARMM¹⁸. First, constraints were applied in regions of predicted α -helix or β -sheet (Fig. 2) to give an extended α -carbon structure in which no turns were built. This structure was then folded, adjusting torsion angles in the regions predicted to be

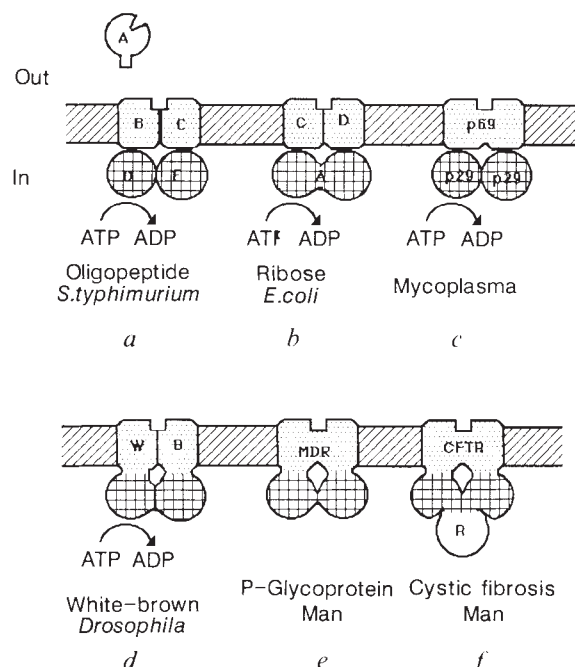


FIG. 1 The organization of ATP-binding cassette (ABC) transporters. The basic transporter requires four distinct domains. Two of these are highly hydrophobic, integral membrane proteins—each of which spans the membrane five or six times by putative α -helices—and are believed to bind the substrate and mediate its passage across the lipid bilayer (stippled). The other two domains couple ATP hydrolysis to transport and are associated with the cytoplasmic face of the membrane (hatched). *a*, In many bacterial systems (for example, the oligopeptide permease of *Salmonella typhimurium*; ref. 29) the four domains are encoded as separate polypeptides (B, C, D, F). These bacterial uptake systems also require the function of a periplasmic substrate-binding protein (A, OppA). However, as mutants which function in the absence of this protein can be isolated³⁰, and as exporters and eukaryotic systems seem to lack an equivalent component, the periplasmic proteins are probably best considered as accessory components and not essential to the mechanisms of transport across the bilayer *per se*. In other systems the domains may be fused into multidomain polypeptides in various ways. *b*, In the ribose system of *Escherichia coli* the two ATP-binding subunits are fused into a single polypeptide (A)³¹. *c*, In the Mycoplasma p69 system the two hydrophobic domains are fused into a single polypeptide³². *d*, The *Drosophila* white and brown loci³³ and the HlyB haemolysin export system of *E. coli*³⁴ each consist of one hydrophobic domain fused to an ATP-binding domain. *e*, *f*, In each of the other eukaryotic systems characterized so far—including MDR (ref. 35), STE6 (refs 36, 37) and CFTR (ref. 23)—all four domains are fused into a single polypeptide. CFTR also has a fifth domain—the R-domain²³—which probably serves a regulatory role. Finally, certain transporter systems appear to lack domains. For example, the histidine and maltose transporters only have a single gene encoding an ATP-binding domain^{38,39}, and the HlyB protein and ProU glycine betaine transporters only appear to have one ATP-binding and one hydrophobic domain^{34,40}. It seems likely that, in such cases, the polypeptides interact as homodimers to provide the four basic domains seen in other systems⁴¹.

turns, to place the α -helices and β -sheets in the same configurations as found in ADK. The predicted secondary structure elements of the ABC proteins could readily be accommodated within the ADK fold without imposing any abnormal constraints on the protein. In contrast, it was not possible to fold the ABC proteins into structures similar to those of PGK, PFK, PK or HK, given the lengths of loops between elements of secondary structure.

In the second stage of modelling, this crudely folded α -carbon model was made to resemble the known ADK structure more closely, using the homology building function of QUANTA to reposition each amino acid in each of the conserved secondary structure elements in the same position in the structure occupied by the equivalent residue in ADK (Fig. 2). Only four short stretches of sequence, designated loops 1, 2, 3 and 4 (Fig. 2), had no direct counterparts in ADK and these were modelled as described in the legend to Fig. 3. Only then were amino acid side chains added to the α -carbon model and energy minimization—constraining main chain positions—used to relieve any unrealistic torsions, poor geometry or contacts between amino acids to give an energetically feasible structure (Fig. 3). Finally, as atomic coordinates for ATP bound to ADK are not available, ATP was docked onto the ADK structure to satisfy NMR nuclear Overhauser effect criteria¹⁹ (Fig. 3) and then added to the ABC model by analogy with its location within ADK.

Our model of the ATP-binding cassette satisfies the general rules of protein folding, although other structures might also fulfill these criteria. However, several lines of evidence support the underlying assumption that the ABC proteins fold in a similar manner to ADK. First, ADK was the only ATP-binding protein that could provide a framework on which the ABC proteins could be modelled without introducing unacceptable constraints. Second, the model was initially derived without considering the nature of the amino-acid side chains (except in so far as they influenced potential secondary structures). When subsequently added to the model, the amino-acid side chains were found to be positioned in a manner consistent with an actual protein structure. Thus, the hydrophobic β -sheets of the Rossmann fold²⁰ were maintained (Fig. 3c), there were no inappropriately buried hydrophilic residues, and patches of hydrophilic residues were confined to the surface of the structure. Furthermore, the model predicts a cleft of dimensions suitable for binding ATP; no unacceptable amino-acid side chains are exposed within the cleft and the two Walker motifs are appropriately positioned. Finally, regions of the ABC proteins that are hypervariable in sequence are exposed at the surface of the structure, and insertions or deletions of amino acids between ABC proteins occur in regions predicted to form loops and would, therefore, not be expected to affect the core structure¹⁷.

The most important differences between the model of the ABC domain and the known structure of ADK are loops 2 and 3. Loop 2 has no equivalent in ADK and, given where it is inserted at the surface of the structure, it seems unlikely to be involved in ATP-binding and hydrolysis; the model implies that loop 2 serves a role specific to the ABC proteins. The fact that loop 3 is so highly conserved in ABC proteins implies that it, too, serves an ABC-specific function rather than being involved in ATP-binding or hydrolysis. Importantly, the regions of ADK equivalent to loops 2 and 3 are those that undergo the greatest conformational changes on binding ATP^{21,22}. We suggest that loops 2 and 3 may be responsible for coupling ATP-dependent conformational changes to the transport process, presumably by an interaction with the hydrophobic membrane domains of the transport systems. The hydrophobicity of loop 2 is consistent with such a role. Similarly, α -helix E, which links loop 2 to the main body of the protein, is a highly conserved structure despite considerable sequence variation between the ABC proteins, consistent with a role in transducing conformational changes to loop 2.

Intriguingly, loops 2 and 3 are precisely where the published cystic fibrosis defects lie. About 70% of Caucasian cystic fibrosis (CF) patients have phenylalanine 508 deleted from loop 2 of the first ATP-binding domain of the CF protein (CFTR) (ref. 23). The adjacent amino acid, isoleucine 507, is deleted in some other patients (M. Super, personal communication); a cluster of mutations in loop 3 has been reported by Cutting *et al.*²⁴. Significantly, temperature-sensitive mutations selected in the related FtsE cell division protein of *E. coli*²⁵ are all located in loops 2 and 3 (D.R.G., unpublished data). The fact that CF mutations are clustered in loops 2 and 3 argues against the suggestion²³ that CF patients are defective in binding or hydrolysing ATP. Furthermore, any mutation that prevents ATP binding would inactivate the protein and have the same phenotype as a null mutation; although frame-shift and nonsense mutations in the CF gene have been identified^{24,26}, spontaneous mutations leading to CF arise infrequently, suggesting that individuals homozygous for null mutations are either not viable or do not exhibit a strong phenotype. We therefore suggest that CF is a

consequence of a specific alteration in the function of the CFTR protein, for example its substrate specificity, rather than simply a loss of CFTR activity. Specifically, CF mutations may alter interactions between the ATP-binding cassette and the membrane-associated domains of the transport system. This model would predict that other CF mutations (excluding null mutations) will alter loops 2 or 3 of either of the two ATP-binding domains of the CFTR protein, modify sequences in the ATP-binding domains that transmit conformational changes to loop 2, or affect regions of the hydrophobic membrane domains with which the ATP-binding domains interact.

It is well established that CF patients have altered chloride-channel activity (reviewed in ref. 27). On the basis of our understanding of ABC transporters, it seems unlikely that the CFTR protein is the chloride channel itself. First, ABC-transporters accumulate substrates against concentration gradients, requiring ATP hydrolysis, but the chloride channels do not. Second, ABC transporters are unidirectional (either import or export), whereas the chloride channel can operate in either

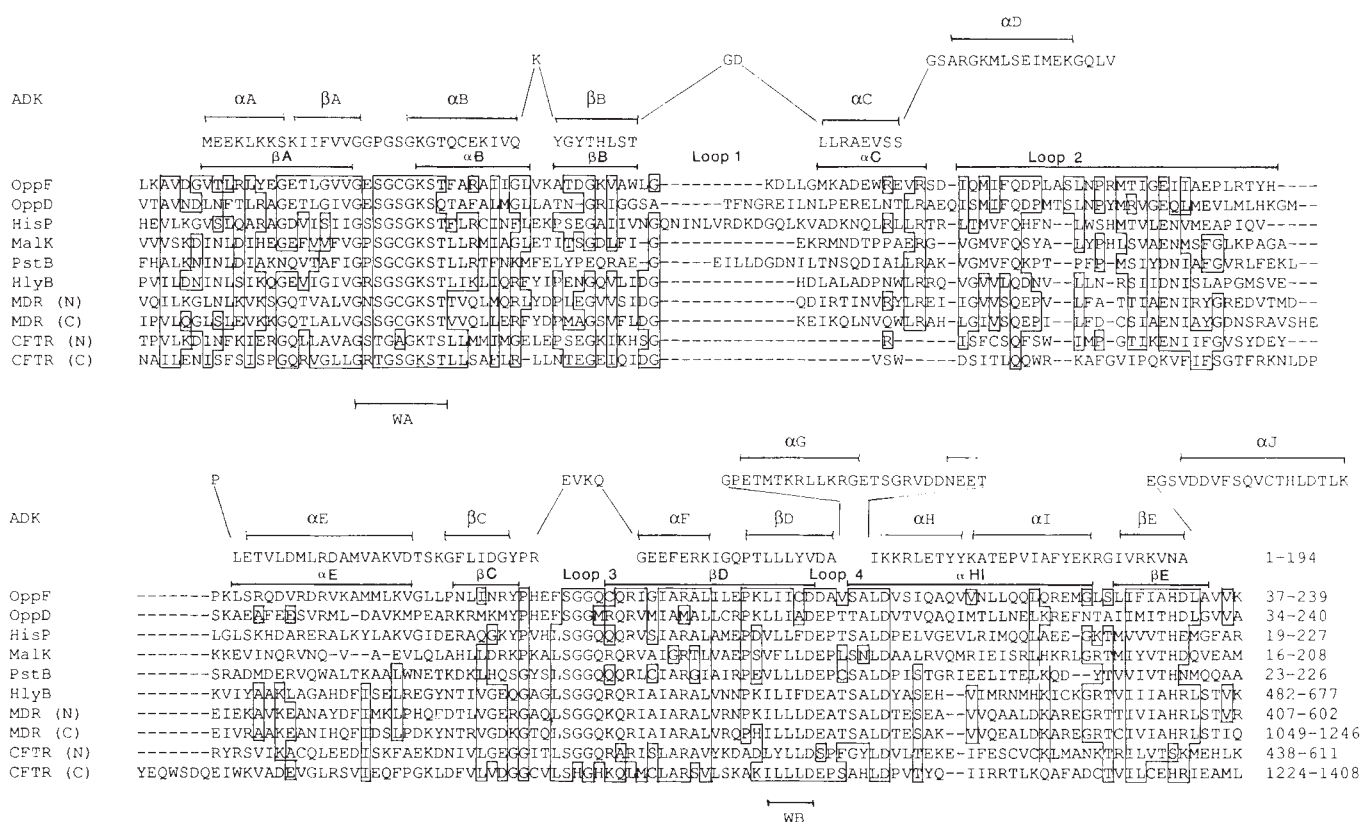
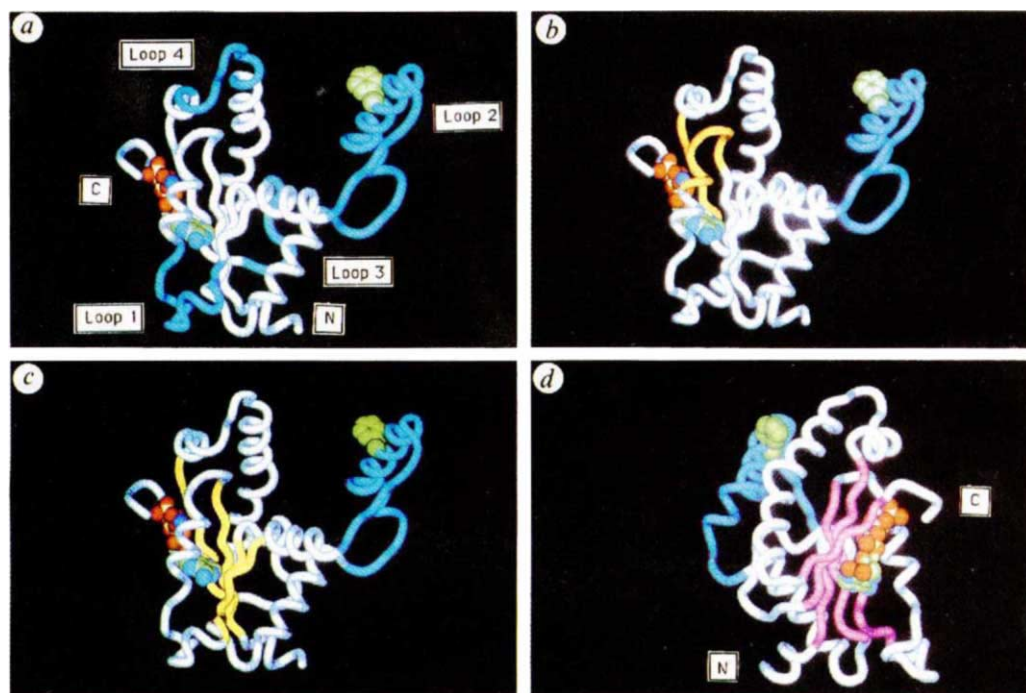


FIG. 2 Primary and predicted secondary structures of ABC-proteins. A number of ABC proteins are aligned to show sequence conservation (shaded). The locations of the predicted secondary structure motifs used in modelling are indicated. Each element is named according to the equivalent element in ADK. The predictions were generated using the Chou-Fasman algorithm and modified in light of a consensus derived from multiple predictions on other ABC proteins. Although such algorithms are not entirely reliable, for such a large family of related proteins confidence is increased for consensus secondary structures that are predicted in the same position in each protein even where the primary sequences differ. In addition, the same algorithm was used as a control to predict secondary structure elements of ADK (porcine); although ADK was part of the database used in formulating the secondary structure prediction methods, it was encouraging that the predicted structures were consistent with those determined by crystallography¹³. Many ABC proteins include amino acids at the N- and C-termini, outwith the conserved ATP-binding cassette⁴¹. It is reasonable to assume that these amino acids have no important effect on the structure of the ATP-binding cassette, which must fold in a context-independent fashion, and they were

excluded from the modelling. The amino-acid residues modelled are those shown in the figure, numbered as in the complete primary sequences of the proteins. The ADK sequence is shown above the alignment of the ABC proteins. The ADK structural elements indicated are those determined by crystallography¹³. The ADK sequence is aligned with that of the ABC proteins, although this alignment is based on secondary structure similarities and not on primary sequence conservation; apart from the Walker motifs⁴, which are labelled WA and WB, ADK shares little primary sequence similarity with the ABC proteins. The lower line of ADK sequence represents residues which, in the final model, were replaced directly by aligned residues from the ABC proteins. Numbering of the ADK amino acids is according to the AK1 designation²¹. Residues in the upper line have no direct counterpart in the model of the ABC proteins. The modelling of these loops is discussed in the text and in the legend to Fig. 3. Residue Phe 508, deleted in many CF patients²³, is indicated. The amino-acid sequences were from the following sources: OppD and OppF (ref. 29); HisP (ref. 38); MalK (ref. 39); HlyB (ref. 34); PstB (ref. 42); Mdr (ref. 43); CFTR (ref. 23).

FIG. 3 Tertiary structure model of the ABC cassette. The four panels show different views of the model structure with different motifs highlighted. The core of the model, based on the structure of ADK, was derived as described in the text. Loops 1, 2, 3 and 4 have no direct counterparts in ADK and were modelled separately. Loops 1, 3 and 4 were comparatively easy to model as they had simply to be shortened or enlarged compared with the equivalent loops in ADK, though maintaining the orientation of the flanking secondary structure motifs. The modelling of loop 2 was more difficult as there is no analogue in ADK. Although the point at which this loop emerges from the core structure is defined, the conformation adopted by the loop itself cannot be predicted with any certainty and must be viewed in this light. The loops were selected from a fragment database essentially as described⁴⁴ and docked into the main body of the structure by fragment movement and appropriate adjustment of torsion angles. The residues around the insertion of the loops were standardized using 200–400 steps of steepest descent and 100–200 steps of ABNR minimization. Quanta software provided by Polygen Corporation, Waltham, Massachusetts, USA. The locations of residue Phe 508 in the N-terminal CF cassette, and the bound ATP molecule, are shown in 'space-filling' format. ADK has two nucleotide-binding sites, one for AMP and one for ATP²¹. However, these have not been defined at an atomic level. We assume that only one nucleotide is bound by the ABC proteins and that this is likely to be at the equivalent of the 'AMP' site of ADK²¹. In particular, sequences in ADK that are thought to interact with the bound nucleotide in the 'AMP' site^{13,21} are conserved in the ABC proteins. In contrast, amino acids (for example, Asp 93, Arg 44) in the 'ATP' site of ADK are absent from the ABC proteins. Furthermore, this site in ADK seems to be able to bind ATP¹⁹. Therefore, ATP was docked into the 'AMP' site of



ADK based on NOE criteria obtained for ATP bound at this site¹⁹. Because a conformational change occurs in ADK on binding ATP²¹ it is not possible to be definitive about the precise location of the ATP, but the position shown is consistent with the proposed interactions between ATP and the Walker motif^{4,21}. No gross change in the location of ATP within ADK would satisfy all these criteria. *a*, The regions of the ABC cassette lacking direct counterparts in ADK (loops 1, 2, 3 and 4) are in blue. The relationship of these regions to the primary sequences are shown in Fig. 2. The N- and C-termini are indicated, as are the loops. *b*, The two Walker motifs, characteristic of many nucleotide-binding proteins, are in yellow and loop 2 in blue. *c*, The five β -sheets of the Rossmann fold²⁰ are in yellow. *d*, View of the structure rotated by 90°. The five β -sheets of the Rossmann fold are in pink and loop 2 is in blue.

direction. Third, the chloride channel has a rapid turnover number characteristic of channels and far greater than has been observed for any ABC transporter. Fourth, the characteristics of chloride channels differ between tissues; it is unlikely (although not impossible) that they are the products of a single gene. Finally, the molecular weight of a putative chloride carrier

is much less than that of CFTR (ref. 28). The model presented here provides a basis for addressing the nature of the substrate(s) transported by CFTR, the mechanisms by which a defect in CFTR affects chloride-channel activity, as well as other mechanistic questions relating to this transport superfamily. □

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A cluster of cystic fibrosis mutations in the first nucleotide-binding fold of the cystic fibrosis conductance regulator protein

Garry R. Cutting^{*†}, Laura M. Kasch^{*},
Beryl J. Rosenstein[†], Julian Zielenski[‡],
Lap-Chee Tsui[‡], Stylianos E. Antonarakis^{*†}
& Haig H. Kazazian Jr^{*†}

^{*} Center for Medical Genetics and [†] Department of Pediatrics,
The Johns Hopkins University School of Medicine, Baltimore,
Maryland 21205, USA

[‡] Department of Genetics, The Hospital for Sick Children, Toronto,
Ontario M5G 1X8, Canada

THE gene responsible for cystic fibrosis (CF) has recently been identified and is predicted to encode a protein of 1,480 amino acids called the CF transmembrane conductance regulator (CFTR)^{1,2}. Several functional regions are thought to exist in the CFTR protein, including two areas for ATP-binding, termed nucleotide-binding folds (NBFs), a regulatory (R) region that has many possible sites for phosphorylation by protein kinases A and C, and two hydrophobic regions that probably interact with cell membranes². The most common CF gene mutation leads to omission of phenylalanine residue 508 in the putative first NBF, indicating that this region is functionally important²⁻⁴. To determine whether other mutations occur in the NBFs of CFTR, we determined the nucleotide sequences of exons 9, 10, 11 and 12 (encoding the first NBF) and exons 20, 21 and 22 (encoding most of the second NBF) from 20 Caucasian and 18 American-black CF patients. One cluster of four mutations was discovered in a 30-base-pair region of exon 11. Three of these mutations cause amino-acid substitutions at residues that are highly conserved among the CFTR protein, the multiple-drug-resistance proteins and ATP-binding membrane-associated transport proteins. The fourth mutation creates a premature termination signal. These mutations reveal a functionally important region in the CFTR protein and provide further evidence that CFTR is a member of the family of ATP-dependent transport proteins^{2,5}.

The association between DNA polymorphism haplotypes and specific gene mutations has been established in a variety of autosomal recessive disorders⁶⁻⁹. Therefore, we determined the haplotypes of 155 Caucasian and 43 American-black CF chromosomes using three DNA markers 5' of the CF gene (XV2c, KM19 and Mp6d.9) and one DNA marker in the gene (G2). The three-basepair deletion mutation leading to the omission of a phenylalanine residue at codon 508 ($\Delta F508$; F denotes Phe in the single-letter amino-acid code) was almost exclusively associated with one haplotype (E in Table 1) and accounted for 77% of the mutations on CF chromosomes from our Caucasian patients, similar to the findings of other studies of North American Caucasians^{3,4}. By contrast, only 16 of 43 (37%) CF chromosomes from American-black patients had the $\Delta F508$ mutation, confirming that racial admixture alone does not account for the incidence of CF in this group¹⁰. Six haplotypes are associated with 29 Caucasian CF chromosomes with unknown mutations, that is, without the $\Delta F508$ mutation, 24 of these chromosomes having an A, D or E haplotype (Table 1). Unknown CF mutations in American-black patients are associated with a wider distribution of haplotypes than in Caucasians (Table 1).

An initial panel of 10 Caucasian CF patients having 14 of 35 unknown CF genes shown in Table 1 and representing each haplotype group was selected for nucleotide sequencing⁶. All

of 18 American-black patients with 27 unknown genes were also examined. Exons 9, 10, 11, 12, 20, 21 and 22 have so far been analysed. We found four exon-11 mutations occurring in a 30-base pair region; three cause amino-acid substitutions, whereas the fourth produces a termination codon (Fig. 1a). One mutation was found in 4% of Caucasian CF chromosomes, another occurred in 5% of American-black CF chromosomes, and the remaining two are rare mutations in American-black patients (Table 2). Every mutation was present in at least one relative of each patient. To eliminate the possibility that the missense mutations are normal variants, we analysed normal chromosomes. Because neutral polymorphisms are expected to occur on normal and CF chromosomes with the same haplotype, chromosomes with the same haplotype as that associated with each mutation were selected for analysis from our group of normal chromosomes (148 American black, 135 Caucasian). By this method, we examined the normal chromosomes most likely to have the sequence variation if it were a neutral polymorphism. The missense mutations in exon 11 were exclusive to CF chromosomes and were never associated with normal chromosomes or CF chromosomes with the $\Delta F508$ mutation (Table 3).

The G1,778 $\rightarrow$ A (Ser549 $\rightarrow$ Asn or S549N in the single-letter amino-acid code) mutation was identified on one chromosome from an American-black CF patient and was inherited from the patient's mother. This mutation causes a conservative substitution between uncharged polar amino acids. It is associated with haplotype A, and was not found on 40 Caucasian or 53 American-black normal chromosomes with at least two sites in common with haplotype A (Table 3).

The G1,784 $\rightarrow$ A (Gly 551 $\rightarrow$ Asp; G551D) mutation was discovered on six Caucasian chromosomes, five of which have the same 10-site haplotype D16/18. The sixth occurred on a chromosome which was identical at four sites closest to the gene (D03 haplotype in Table 3) but which differed at more distant sites. So far, this is the second most common CF mutation in Caucasians. It is unlikely that this mutation is a protein polymorphism, as it replaces a neutral with a charged amino acid. Furthermore, the mutation occurs on 4% of Caucasian CF chromosomes in our sample and has not been found on three normal chromosomes with the same 10-site haplotype or on 54 other normal Caucasian chromosomes. In six of seven Caucasian patients (including two siblings) who had this mutation, it was paired with the $\Delta F508$ mutation. Three of these patients, ages 11-13 years, have mild lung disease with normal pulmonary function test results, whereas the other three patients, ages 15-17 years, have moderate-to-severe pulmonary disease. The seventh patient with the G551D mutation, age 31 years, has an unknown mutation on his other CF chromosome and has mild lung disease. All of the patients except one from the sibling pair have exocrine pancreatic insufficiency, requiring supplements of pancreatic enzymes. The range of illness severity and small number of patients precluded a meaningful assessment of the effect of this mutation on phenotype. All patients are of northern European ancestry representing different ethnic groups.

The nucleotide substitution C1,789 $\rightarrow$ T (Arg 533 $\rightarrow$ Stop; R533 Stop) is the first nonsense mutation observed in the CF gene. It occurs at a CG dinucleotide, a 'hotspot' for mutations, and it conforms to the CG $\rightarrow$ TG rule^{16,17}. This mutation was found on two American-black chromosomes having haplotypes identical at eight informative sites suggesting that it has a common origin. It is not known whether a stable truncated CFTR protein is present *in vivo*; but in other disorders, nonsense mutations have been associated with unstable protein products¹⁸. One of the two patients with this nonsense mutation is a genetic compound with the G1,778 $\rightarrow$ A (S549N) mutation (patient 272 in Fig. 1a).

The fourth mutation, a G1,807 $\rightarrow$ A substitution, was found on one chromosome from an American-black patient. This mutation causes a conservative change of Ala 559 $\rightarrow$ Thr (A559T); it is associated, however, with a relatively common four-site

TABLE 1 Haplotype distributions

Haplotype	XV2c <i>TaqI</i>	KM19 <i>PstI</i>	D9 <i>MspI</i>	G2 <i>XbaI</i>	Unk	Caucasian $\Delta F508$	American black Unk $\Delta F508$
A	1	1	1	2	6	0	1 0
B	1	1	2	2	2	0	2 0
C	1	2	1	2	0	0	1 0
D	1	2	2	1	12	5	1 0
E	1	2	2	2	6	92	2 7
F	2	1	1	2	1	0	4 0
G	2	2	1	1	0	0	1 0
H	2	2	2	1	2	1	1 0
I	2	2	2	2	0	6	2 2
Other	-	-	-	-	6	16	12 7
					35(0.23)	120(0.77)	27(0.63) 16(0.37)

Distribution of haplotypes by race on chromosomes bearing the CF gene with an unknown (Unk) mutation or the deletion $\Delta F508$ mutation in the Johns Hopkins database. Parentheses indicate frequency; a dash indicates polymorphism uninformative or unknown; 1 denotes the absence, and 2 denotes the presence, of a restriction site. DNA markers XV2c and KM19 and their associated polymorphisms are described for these populations elsewhere¹⁰. Probes D9 (M_p6d.9) and G2, which detect *MspI* and *XbaI* polymorphic sites respectively, were obtained from Professor R. Williamson^{11,12}. Direct detection of the $\Delta F508$ mutation was performed by polymerase chain reaction (PCR) amplification of genomic DNA using primers C16B and C16D followed by vacuum blotting of amplified DNA to nitrocellulose filters and hybridization with radioactively labelled oligonucleotides of either normal sequence or deletion F508 sequence as previously described³.

TABLE 2 Mutations detected in exon 11 of the CF gene

Mutation	CF chromosome	Restriction-enzyme site change caused by mutation
Nucleotide	Amino acid	Racial origin
G1,778 → A	Ser 549 → Asn	American black
G1,784 → A	Gly 551 → Asp	Caucasian
C1,789 → T	Arg 553 → Stop	American black
G1,807 → A	Ala 559 → Thr	American black
		Haplotypes (no. of chromosomes)*
		A15 (1)
		D16/18 (5) D03 (1)
		I12 (1) Ii06 (1)
		F (1)
		None

* Haplotype codes: first capitalized letter, four-site (XV2c, KM19, D9 and G2) haplotype shown in Table 1; numbers following the letter, extended haplotype (7C22, MET, D7S8); i, incomplete haplotype informative for at least three of the sites in the four-site haplotype.

† PCR amplification of genomic DNA using exon-11 primers 11i-5' and 11i-3' (see Fig. 1a) produces a 425-base-pair (bp) fragment. *DdeI* digestion of DNA amplified from normal exon-11 sequence creates two fragments of 174 and 251 bp, whereas DNA amplified from exon 11 sequence containing this mutation will not be cut with *DdeI*.

‡ *HincII* digestion of DNA amplified from normal exon-11 sequence (425 bp) produces fragments of 186 and 239 bp. The Gly 551 → Asp and Arg 553 → Stop mutations both remove the normal *HincII* recognition site.

§ DNA amplified from normal exon-11 sequence (425 bp) cannot be cut with *MboI*, whereas digestion of DNA amplified from exon-11 sequence containing this mutation will create two fragments of 182 and 243 bp.

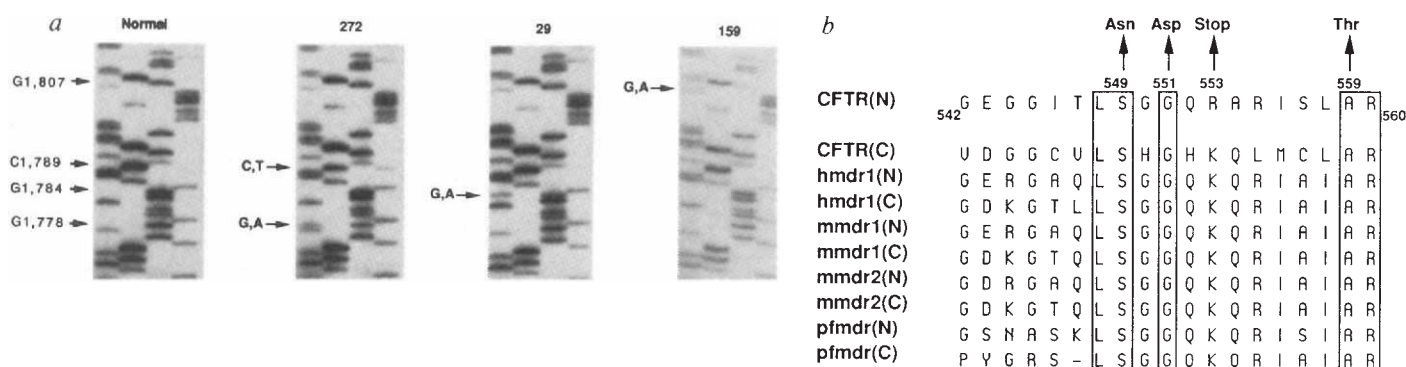
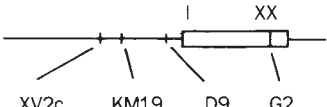


FIG. 1 a, Direct sequence analysis of PCR-amplified genomic DNA from exon 11 of CFTR using the primer 11i-5'. The order of nucleotides is A, C, G, T. Normal sequence extending from nucleotide position 1,769 to 1,816 is shown beside sequence obtained from patients 272, 29 and 159. Patient 272 has exon 11 mutations on each chromosome, G1,778 → A (G,A) and C1,789 → T (C,T); patient 29 has the mutation G1,784 → A; patient 159 has the mutation G1,807 → A. b, Alignment of predicted amino-acid sequence (single-letter code) from residues 542-560 in exon 11 of the first NBF with conserved regions in the second NBF of CFTR and similar regions in four members of the multiple drug resistance (mdr) protein family. Positions of the amino-acid substitutions and stop signal found in this study are shown. Amino acids conserved in all 10 sequences are boxed. Adapted from ref. 2. N, N terminal; C, C terminal; h, human; m, mouse 1 and 2; pf, *Plasmodium*

falciparum mdr proteins.

METHODS. PCR amplification was performed as previously described¹³ using oligonucleotide primers 11i-5' (5'-CAACTGTGGTTAAAGCAATAGTGT-3') and 11i-3' (5'-GCACAGATTCTGAGTAACCAAT-3') selected from intron sequences flanking 5' and 3' of exon 11 of the CFTR gene. About 500 ng genomic DNA from peripheral lymphocytes of each subject was amplified using 2 µl of a 10 µM solution of each primer described above in a total volume of 100 µl containing *Taq* polymerase buffer (50 mM KCl, 10 mM Tris buffer, pH 8.3, 1.5 mM MgCl₂, 0.01% (w/v) gelatin), 0.02 µM each 2'-deoxynucleotide 5'-triphosphate (Pharmacia) and 2.5U *Taq* polymerase (Cetus). Amplification was performed by 30 cycles of annealing at 58 °C for 30 s, extension at 72 °C for 1 min and denaturing at 94 °C for 30 s. Sequencing of DNA amplified from genomic DNA was performed as described^{14,15}.

TABLE 3 DNA polymorphism haplotypes associated with the exon-11 missense mutations

Haplotype*					CF chromosomes†			Normal chromosomes‡		
	XV2c T	KM19 P	D9 M	G2 X	G1,778 → A	Normal at 1,778 Caucasian	Black	G1,778 → A	Normal at 1,778 Caucasian	Black
Mutation G1,778 → A										
A15	1	1	1	2	1	0	0	0	0	0
A	1	1	1	2	0	5§	0	0	32	6
1c	—	1	1	2	0	1	4	0	0	29
c	—	—	1	2	0	0	3	0	8	18
Other	—	—	—	—	0	27	19	0	40	40
					1	33	26	0	80	93
Mutation G1,784 → A										
D16/18	1	2	2	1	5	0	0	0	3	0
D03	1	2	2	1	1	5	0	0	1	0
D	1	2	2	1	0	1	1	0	7	1
Other	—	—	—	—	0	21	26	0	46	35
					6	27	27	0	57	36
Mutation G1,807 → A										
F	2	1	1	2	1	1	3	0	ND	8
1c	—	1	1	2	0	7	4	0	ND	17
c	—	—	1	2	0	0	3	0	ND	2
Other	—	—	—	—	0	22	16	0	ND	9
					1	30	26	0		36

Map at the top shows the relative positions and approximate distances between the markers XV2c, KM19, D9 and G2 relative to the 250-kilobase CF gene, which is shown as a box^{3,11,12}. Roman numerals denote exons 1 and 20 respectively.

* Extended haplotypes include 7C22, MET and D7S8 sites which are not shown (see Table 2); 1 indicates the absence and 2 the presence of a particular site; —, site is different or uninformative. Enzyme abbreviations: T, *TaqI*; P, *PstI*; M, *MspI*; X, *XbaI*.

† CF chromosomes shown do not have the $\Delta F508$ mutation. Fifty-six Caucasian and nine American-black chromosomes with the $\Delta F508$ mutation did not have the G1,778 → A or G1,784 → A mutations and twenty Caucasian and nine American-black chromosomes with the $\Delta F508$ mutation did not have the G1,807 → T mutation. Fifty Caucasian CF and twenty-six black CF chromosomes were directly sequenced in each case.

‡ Normal Caucasian chromosomes are from parents and/or siblings of CF patients and are therefore non-CF-gene bearing. Normal black chromosomes are either non-CF-gene-bearing chromosomes from healthy family members or chromosomes from black patients heterozygous for sickle-cell anaemia or β -thalassaemia (CF carrier frequency in American blacks is 1 in 65 persons¹⁰). Normal chromosomes with four-site haplotypes (XV2c, KM19, Mp6d.9 and G2) identical to the mutation-bearing chromosomes were examined whenever possible. But two- or three-site haplotypes, which included the intragenic marker G2 and the closest 5' markers (Mp6d.9 ± Km19), were also employed. Screening of normal chromosomes and additional Caucasian CF chromosomes for each mutation was as follows: PCR amplification of exon 11 followed by *DdeI* digestion to detect the G1,778 → A mutation or *MboI* digestion to detect the G1,784 → A mutation or direct sequencing to detect the G1,807 → A mutation (Table 2). ND, not determined.

§ DNA from only five of the six Caucasian patients with an unknown mutation associated with haplotype A was available.

haplotype (F) in the American-black population. Direct sequencing of eight normal chromosomes with the same four-site haplotype, 19 other normal chromosomes with at least two sites in common with haplotype F, and nine additional normal chromosomes from American-black sickle-cell or β -thalassaemia carriers did not reveal this mutation.

The CF gene was identified solely by its location in the human genome¹. Little is known of the function of its protein product except by analogy with well-characterized proteins that have similar amino-acid sequences². Nucleotide sequencing of six other exons of the CF gene (exons 9, 10, 12, 20, 21 and 22) has identified only isolated mutations (G. C., unpublished results). The four mutations described here occur in a 13-amino acid segment (codons 548–560) of the putative first NBF in the CFTR protein, indicating that this region is functionally important. This 13-amino acid segment is highly conserved with similar regions of other membrane-associated transport proteins². Five amino acids in this region are completely conserved in comparable regions from the multiple-drug-resistance proteins, indicating that these positions are probably crucial to protein function (Fig. 1b). The amino-acid substitutions described in this study

occur at three of the five completely conserved residues. Moreover, the substitutions occur at the three most conserved residues in this region among CFTR and 14 other membrane-associated proteins that bind ATP (see in ref. 2). Therefore, the location of this cluster of mutations supports the theory that the CFTR protein is a member of the superfamily of ATP-dependent transport proteins^{2,5}. □

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No evidence for expression of the insulin-regulatable glucose transporter in endothelial cells

Jan W. Slot*, Richard Moxley†, Hans J. Geuze* & David E. James‡

* Department of Cell Biology, Medical School, University of Utrecht, 3584 CX Utrecht, The Netherlands

† Department of Neurology, University of Rochester, Rochester, New York 14642, USA

‡ Department of Cell Biology and Physiology, Washington University School of Medicine, St Louis, Missouri 63110, USA

A MAJOR effect of insulin is to increase glucose transport in muscle and fat^{1,2}. A family of genes encoding distinct mammalian glucose transporters has recently been elucidated^{3–9}. One of these, the insulin-regulatable glucose transporter (IRGT)^{3,10}, is primarily expressed in muscle and fat, tissues that exhibit insulin-dependent glucose transport. Insulin promotes glucose transport in these tissues by stimulating movement of the glucose transporter from an intracellular location to the plasma membrane^{3,10–14}. Recent studies¹⁵, however, suggest that an additional effect of insulin in these tissues may be the facilitation of glucose transport, presumably across capillary endothelium. This hypothesis is based on the localization of the IRGT in endothelial cells specific to muscle and adipose tissue. We report here, however, on morphological and biochemical studies using several different IRGT-specific antibodies in which we could not reproduce these results.

Immunohistochemical analyses were performed using a monoclonal (1F8) and two polyclonal (anti-IRGT₁₂ and anti-IRGT₁₉) antibodies against the IRGT. The monoclonal antibody was produced by immunization of mice with intracellular vesicles from rat adipocytes¹⁰. Anti-IRGT₁₂ and anti-IRGT₁₉ are specific for synthetic peptides encompassing the last 12 and 19 amino acids respectively, of the IRGT C terminus. Light microscopy of cardiac muscle from non-stimulated rats, immunolabelled with anti-IRGT antibodies (Fig. 1a,b) indicated pronounced labelling of intracellular structures close to the nuclei (n) and near the lateral cell surface of myocytes (arrow-heads). These are areas where the regular myofibril pattern is disturbed by accumulated cell organelles, mostly mitochondria. In addition, some labelling was associated with the Z-lines in cardiac myocytes. In skeletal muscle (Fig. 1c,d), labelling of the IRGT was confined to regions at the cell borders of the myocytes. No clear labelling of the Z-lines was observed in these cells. Changes in the labelling pattern in either cardiac or skeletal muscle following insulin stimulation were not as obvious as in renal brown adipose tissue. In brown adipocytes from insulin-treated rats, IRGT labelling was evident along the entire cell surface (Fig. 1e,f), including the domains facing the blood capillaries (c) and the borders between the adipocytes (arrow-heads). Most of the adipocyte surface-labelling could be assigned to the plasma membrane by electron microscopy (Fig. 2). Some labelling was also associated with intracellular structures (Fig. 1e, double arrows). In brown adipocytes from non-insulin treated rats, the cell surface was devoid of IRGT-labelling and the transporter was localized primarily to an intracellular domain (results not shown). These observations are compatible with the current view that the IRGT moves from an intracellular

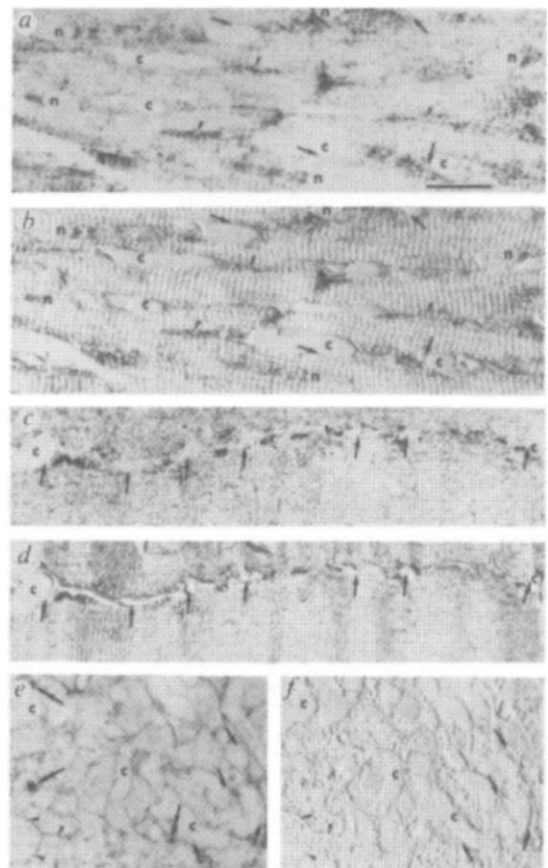


FIG. 1 Distribution of the IRGT in muscle and adipose tissue. Shown here are light micrographs of gold labelling in cryosections visualized by silver enhancement (a, c and e) and the Nomarski image to provide the corresponding structural information (b, d and f). a, b, Cardiac muscle, non-stimulated, labelled with anti-IRGT₁₉; c, d, skeletal muscle, non-stimulated, labelled with anti-IRGT₁₂; e, f, brown adipose tissue, insulin-stimulated, labelled with monoclonal antibody 1F8. Blood capillaries are marked by C. Further marking is explained in the text. Magnification is similar for a–f. Bar in a, 20 μ m. METHODS. Cardiac and skeletal (soleus) muscle were obtained from ~100 g Sprague Dawley rats following perfusion fixation with 0.1 M phosphate buffer (pH 7.4) containing 2% paraformaldehyde and 0.2% glutaraldehyde. Insulin-stimulated rats were fasted overnight and injected with glucose and insulin (1 g glucose, 8 U insulin in 3 ml saline per kg body weight, i.p.), 30 min before fixation. Nonstimulated rats received a saline injection (3 ml saline per kg, i.p.) instead. Renal brown adipose tissue was prepared under identical conditions, except that rats were kept at 4 °C (4 h) before fixation in order to reduce intracellular triglyceride levels. Comparative studies performed in animals not incubated at 4 °C indicated that this treatment did not affect the distribution of the IRGT (data not shown). Tissues were kept for 2 h at 20 °C in fixative before the preparation of semi-thin cryosections (~300 nm). Sections were incubated with antibodies as previously described. Three different anti-IRGT antibodies were used for immunolabelling: two rabbit polyclonal antibodies against either a 12-amino-acid peptide³ (anti-IRGT₁₂), or a 19-amino-acid-peptide (anti-IRGT₁₉) encompassing the C terminus of the IRGT sequence, or a monoclonal antibody (1F8) raised against the rat adipocyte IRGT¹⁰. The anti-IRGT₁₂ and 1F8 were affinity-purified using a protein A Sepharose affinity matrix (BioRad) and used for labelling at concentrations of 5 and 2.5 μ g ml⁻¹ respectively. The anti-IRGT₁₉ was affinity-purified using the peptide antigen conjugated to acrylamide beads (BioRad) and used for labelling at a concentration of 1–5 μ g ml⁻¹. Labelling with the polyclonal antibodies was detected by light microscopy following incubation with swine anti-rabbit IgG, and protein A/gold as previously described³, and immunolabelling with monoclonal antibody 1F8 was visualized following incubation with rabbit anti-mouse IgG and protein A gold. In control sections (not shown), incubations were performed with either a non-specific antibody, or with anti-IRGT₁₂ and anti-IRGT₁₉ mixed 1 h before use with a 10-fold molar excess of the 12- or 19-amino-acid peptide antigen, respectively. In each case the labelling pattern observed in IRGT-labelled sections was completely absent in the controls.

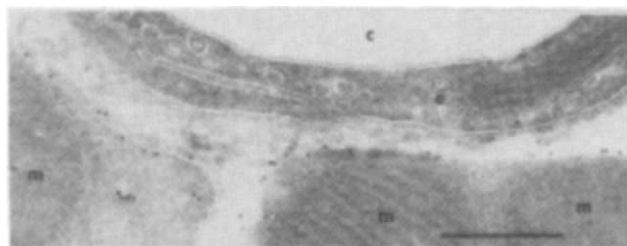


FIG. 2 Electron micrograph of a thin brown adipose tissue cryosection¹⁹ from an insulin-treated rat, immunolabelled with anti-IRGT₁₉ followed by protein A gold (10 nm markers). Fragments of adipocytes with gold labelling along the plasma membrane are shown below. m, Mitochondria. An endothelial cell (e) that lines the capillary space (c) is not significantly labelled. In 5 sections from 5 different insulin-treated rats, we located at low magnification 5–10 cross-sectioned capillaries. Quantitation of gold to within 20 nm from the plasma membrane of endothelial cells and the surrounding plasma membrane of the adipocytes, indicated that the labelling density over luminal and adluminal membranes was $5.2 \pm 2.4\%$ (s.d.) and $6.5 \pm 2.7\%$, respectively, of the gold labelling density in association with the corresponding adipocyte membrane. Bar, 0.5 μm .

region to the cell surface following insulin administration *in vivo*^{3,10–12}.

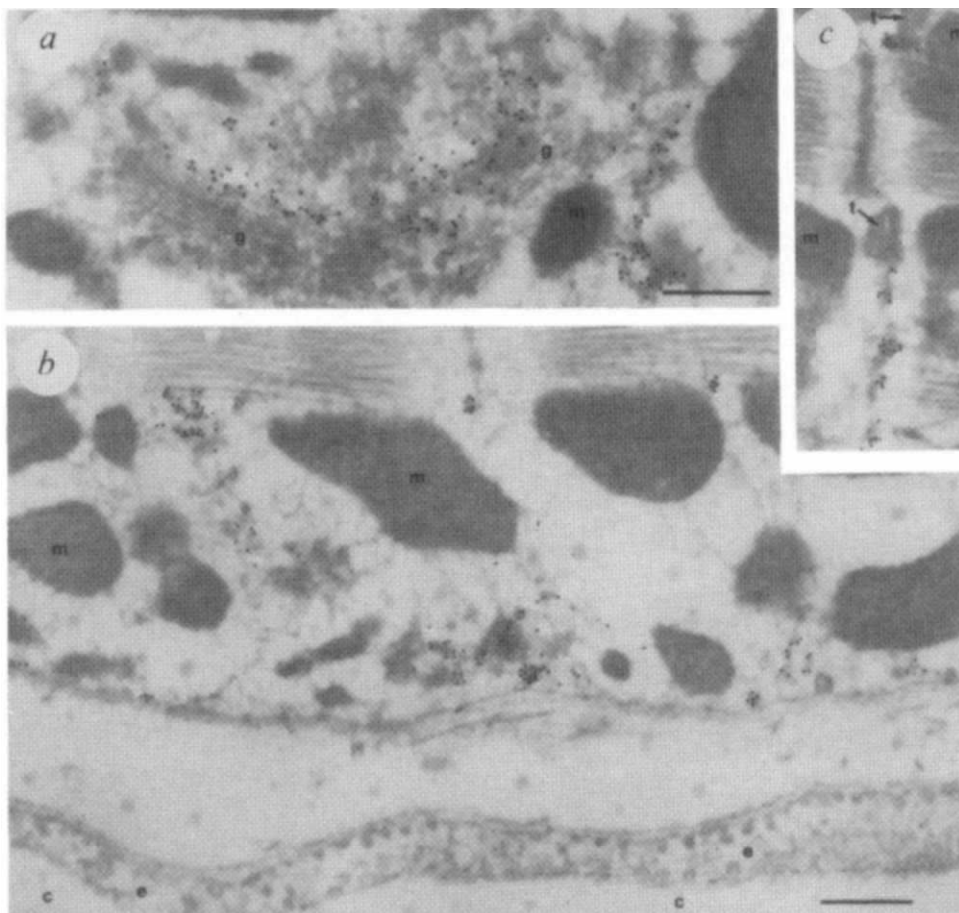
At present we are performing an extensive electron microscopic analysis to characterize the distribution of the IRGT in brown adipose tissue and muscle from animals that have been treated with saline or insulin. Preliminary results have shown that the main intracellular location of the IRGT in adipocytes, as well as in myocytes, is in characteristic small tubular and vesicular structures. As in the cardiac muscle cell (Fig. 3, a–c), many of these labelled structures occur in large agglomerations that are associated with the Golgi complex, representing the trans Golgi reticulum (TGR in Fig. 3a). In addition, many of the labelled vesicles and tubules are scattered throughout the cytoplasm, often in close proximity to the cell surface (Fig. 3b) and, only in the cardiac myocyte, at the Z-line level often near the transverse (T-) tubules (Fig. 3c).

The reactions observed were essentially the same for the three anti-IRGT antibodies, except that 1F8 occasionally gave some spurious reactions (see below). No reaction was observed in control sections incubated with nonspecific antibodies or with anti-IRGT polyclonal antibodies, when preincubated with the

appropriate peptides. The reactivity of 1F8 was quenched after incubating that antibody with one of the peptides, indicating that the epitope of the monoclonal may be at the C-terminal tail of the IRGT as well.

We have observed labelling of the IRGT only in adipocytes and myocytes, but not in other cell types such as endothelial cells (Figs 1, a–f, arrows; 2 and 3b). Quantitation of gold particles in IRGT-labelled brown adipose tissue sections of insulin-treated animals indicated that the labelling density at the surface of endothelial cells was only 5–7% of that associated with the cell surface of adipocytes (Fig. 2). These data are in direct contrast to a recent study using monoclonal antibody 1F8 (ref. 15) in which intense labelling of the endothelial cells specific to muscle and fat tissue and only poor labelling of the corresponding myocytes or adipocytes were reported. To clarify this anomaly, we have isolated endothelial cells from rat epididymal fat pads and have been unable to detect the IRGT in these isolated cells by biochemical means, using the anti-IRGT antibodies employed in this study. In contrast, thrombomodulin, an endothelial cell specific marker¹⁶, was highly enriched in our endothelial cell fraction but undetectable in the adipocyte frac-

FIG. 3 Electron micrographs showing the IRGT distribution in cardiac muscle from a nonstimulated rat. The section was prepared as for Fig. 2, except that anti-IRGT₁₉ incubation was followed by swine anti-rabbit IgG and then protein A/gold (15 nm marker). a, IRGT is abundant in a tubulo-vesicular system, presumably the TGR, near the Golgi complex (g). b, Similar labelled elements occur in small groups or isolated, near the cell surface; c, localization in the cytoplasm between the myofibrils, most frequently at the Z-line level of the sarcomeres close to the surface connected T-tubules (t). No noticeable labelling is present in the endothelial cell (e). Capillary space, c. Bar, 0.5 μm ; magnification in c as in b.



tion. All immunoreactive IRGT present in adipose tissue was detected in the isolated adipocyte fraction (Fig. 4).

We conclude that the level of expression of the IRGT in endothelial cells is insignificant compared with that observed in myocytes and adipocytes. Using three different antibodies we have consistently observed negligible labelling of endothelial cells in skeletal muscle, heart and adipose tissue of five non-insulin-treated and five insulin-treated rats, except for an occasional, spurious reaction with 1F8. We observed other faulty reactions with 1F8, for instance in nuclei of all cell types and in the lumen of myocyte sarcoplasmic reticulum. These observations are at odds with the apparent cytosolic orientation of the epitope for 1F8. These spurious reactions, including those observed in endothelial cells, were not consistently present in 1F8-labelled tissue sections and never in the immunoblotting experiments. Moreover, this aberrant labelling pattern was never observed using polyclonal antibodies. We were able to compete out both 1F8 and the polyclonal antibody immunolabelling of cells when the antibodies were preincubated with a 10-fold molar excess of the IRGT C-terminal peptide. In fact this was even evident with respect to the endothelial cell and nuclear staining of 1F8. This might indicate that the spurious labelling observed with 1F8 is due to weak cross reaction with other than IRGT molecule(s). Most probably this can only result in low stringent binding, so that minor variations in the reaction conditions can explain why the cross reaction is only sometimes manifest in immunolabelled preparations, and why these spurious 1F8 reactions are not reported from immunoprecipitation¹⁰ or immunoblotting (Fig. 4) studies. These observations suggest that localization of the IRGT using only 1F8 may be difficult to interpret and we suspect that the previous data¹⁵ may largely reflect the aberrant labelling pattern of the monoclonal antibody.

Our data indicate that it is the permeability of the myocyte or adipocyte membrane that limits entry of glucose into these cells. Previous studies have shown quite convincingly that in cardiac muscle there is no significant difference in transcapillary flux of L- compared with D-glucose^{17,18}. Thus, the existence of a transporter to facilitate the movement of glucose out of the

endothelium, at least in cardiac muscle, would seem to be redundant. We conclude that, in muscle and adipose tissue, the IRGT is primarily expressed in myocytes and adipocytes, respectively. In these cells the transporter is enriched in the trans Golgi reticulum and in small vesicles and tubules scattered throughout the cytoplasm. These sites of IRGT seem to be in an insulin-regulated equilibrium with the plasma membrane. □

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Suppression of experimental glomerulonephritis by antiserum against transforming growth factor β 1

Wayne A. Border*, Seiya Okuda*, Lucia R. Languino†, Michael B. Sporn‡ & Erkki Ruoslahti†

* Division of Nephrology, University of Utah School of Medicine, Salt Lake City, Utah 84132, USA

† Cancer Research Center, La Jolla Cancer Research Foundation, 10901 N. Torrey Pines Road, La Jolla, California 92037, USA

‡ Laboratory of Chemoprevention, National Cancer Institute, NIH, Bethesda, Maryland 20892, USA

GLOMERULONEPHRITIS is an inflammation of the kidney characterized by the accumulation of extracellular matrix within the damaged glomeruli¹⁻⁴, impaired filtration and proteinuria. In its progressive form, the disease destroys kidney function leading to uraemia and death, unless dialysis therapy or kidney transplantation is available. The pathogenesis of glomerulonephritis is incompletely understood, but the eliciting factor is thought often to be an immunological injury to mesangial and/or other resident cells in the glomeruli^{5,6}. We have used an animal model of acute mesangial proliferative glomerulonephritis^{7,8} to show that this disease is associated with increased production and activity of transforming growth factor β 1 (TGF- β 1)⁹, an inducer of extracellular matrix production¹⁰⁻¹⁷. Here we report that administration of anti-TGF- β 1 at the time of induction of the glomerular disease suppresses the increased production of extracellular matrix and dramatically attenuates histological manifestations of the disease. These results provide direct evidence for a causal role of TGF- β 1 in the pathogenesis of the experimental disease and suggest a new approach to the therapy of glomerulonephritis.

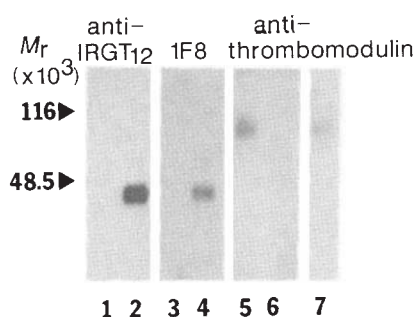
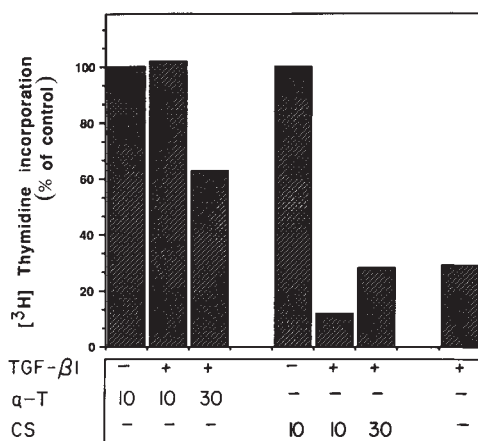


FIG. 4 Distribution of the IRGT (lanes 1-4) and thrombomodulin (lanes 5-7) in endothelial cells (lanes 1, 3 and 5) and adipocytes (lanes 2, 4 and 6) isolated from rat epididymal fat pads. Aliquots (10 μ g total protein) of the homogenates were immunoblotted with either anti-IRGT₁₂ (lanes 1, 2), 1F8 (lanes 3, 4) or anti-thrombomodulin²¹ (lanes 5, 6, 7). An aliquot of purified rat thrombomodulin was also immunoblotted as a reference (lane 7). Relative molecular weights (M_r) are indicated on the left.

METHODS. Epididymal fat pads were dissected from male rats (150 g) and digested with collagenase for 45 min at 37 °C as previously described^{11,12}. Endothelial cells were prepared essentially as described²⁰. Following collagenase digestion, cells were passed through a 250- μ m pore nylon filter. The fat cells were allowed to float to the surface and removed. The remaining filtrate was centrifuged at 400g (5 min). The pellet was washed with Krebs Ringer buffer and passed through a 20- μ m pore nylon filter. The clumps of endothelium that remained attached to the filter were washed in buffer and recentrifuged. Inspection of this fraction by light microscopy revealed an identical morphology to that reported previously for microvascular endothelium with little contamination from cells of other origin²⁰. From 50 rats we isolated ~250 μ g of endothelial cell protein.



Glomerular mesangial cells express a Thy-1.1 epitope on their surface⁷. Injection of antithymocyte serum into an animal produces a dose- and complement-dependent selective injury to mesangial cells resulting in acute mesangial proliferative glomerulonephritis^{7,8}. The glomerular lesion is characterized by expansion of the mesangial matrix and hypercellularity, resembling the morphological features of human mesangial proliferative glomerulonephritis.

Induction of glomerulonephritis in rats with a single injection of antithymocyte serum was followed by treatment with an injection of either anti-TGF-β1 or control sera. The anti-TGF-β1 serum was prepared by immunizing a rabbit with a synthetic peptide containing residues 78–109 from human mature TGF-β1; the cyclized form of this peptide elicits an antiserum capable of inhibiting binding of TGF-β1 to cells¹⁸. The specificity of the

FIG. 1 Inhibition of TGF-β1 activity by antibodies. A mink lung epithelial cell assay that measures growth inhibition by TGF-β (ref. 30) was used to assay the ability of anti-TGF-β antiserum to neutralize its activity. Dilutions of 1:10 (10) and 1:30 (30) of anti-TGF-β1 serum (a-T) inhibited the action of TGF-β1 (TGF-β1) added to the cultured cells. Control antiserum (CS) had no effect (+, added; -, not added).

METHODS. Anti-TGF-β1 serum was prepared against a synthetic peptide from residues 78–109 (ref. 18) of human mature TGF-β1. The peptide was synthesized in an Applied Biosystems solid phase peptide synthesizer and purified by HPLC. The rabbit was immunized subcutaneously with 2 mg per injection of the peptide which was mixed with 0.5 mg methylated BSA³¹ and emulsified in Freund's complete adjuvant. The injections were generally given 4 weeks apart and the rabbit was bled about 1 week after the second and every successive injection. The antisera used in this work had a titre (50% binding) of 1:6,000 in radioimmunoassay, bound to TGF-β1 in immunoblots and inhibited the induction of proteoglycan synthesis caused by TGF-β1 in cultured mesangial cells (results not shown). Growth inhibition was assayed by adding to mink lung epithelial cell cultures 3 ng ml⁻¹ human platelet TGF-β1 (Calbiochem) which had been preincubated with dilutions of 1:10 or 1:30 of heat-inactivated (56 °C, 30 min) anti-TGF-β1 serum or with identical dilutions of a control antiserum prepared against an unrelated peptide (from the cytoplasmic domain of the α₄-subunit of an integrin), followed by an assay for [³H]thymidine incorporation³⁰.

antiserum was established as described in the legend of Fig. 1 and it can neutralize the activity of purified TGF-β1 in a bioassay (Fig. 1).

The effects of the anti-TGF-β antiserum and control sera on the glomerulonephritis model were evaluated by testing 10 animals in each group in three different experiments. We used histological evaluation of glomerular extracellular matrix accumulation as a measure of disease activity, because it is a characteristic feature of acute glomerulonephritis^{1–4}.

Figure 2 shows a comparison of the microscopic appearance of representative glomeruli, and measurement of the glomerular extracellular matrix is shown in Fig. 3. The glomeruli contain much less extracellular matrix material in the anti-TGF-β1-treated groups than in the groups receiving control serum. Immunofluorescent staining of the tissues with antibodies

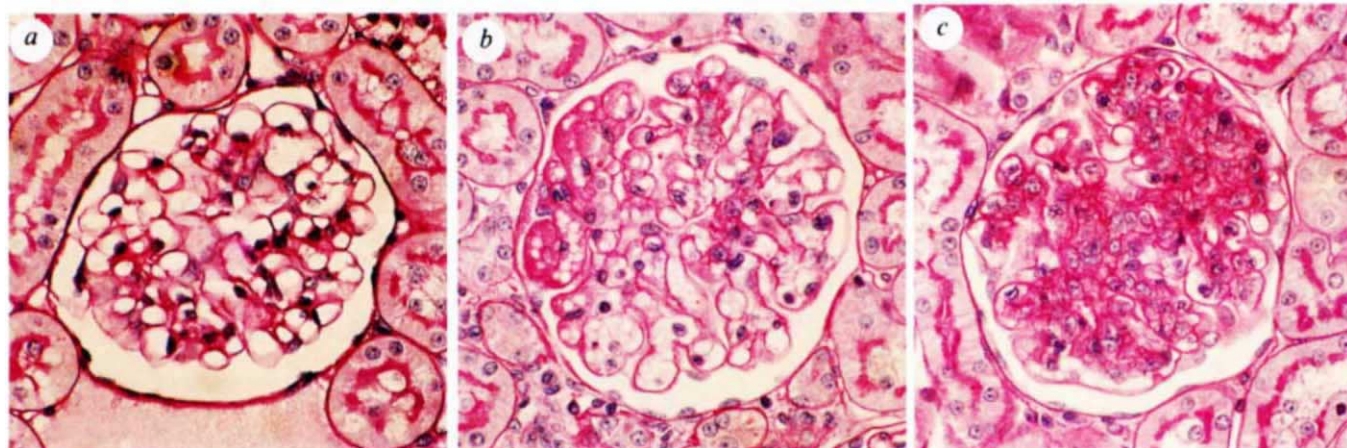


FIG. 2 Histological analysis showing pathological changes in glomeruli of glomerulonephritic kidneys. Micrographs showing periodic acid-Schiff staining of glomeruli. *a*, Staining of the basement membrane and extracellular matrix in a normal glomerulus. *b*, From a rat on day 7 after the injection of antithymocyte serum. This rat received injections of 1 ml rabbit anti-TGF-β1 on six successive days, starting on the day of the antithymocyte injection. *c*, From an animal that received control serum under a similar regimen. A striking increase in extracellular matrix is seen as the reddish-pink amorphous material filling most of the glomerulus in *c*; *b* shows a clear effect of anti-TGF-β1 in preventing the increase in glomerular extracellular matrix that occurs on day 7 after injection of antithymocyte serum. Magnification, $\times 500$.

METHODS. Antithymocyte serum was produced by immunizing New Zealand white rabbits with 1×10^6 rat thymocytes in complete Freund's adjuvant, followed by boosting with 1×10^6 thymocytes given intravenously 2 and 4

weeks later⁹. The serum was absorbed three times each with packed rat erythrocytes and rat liver powder to remove nonspecific reactivity. Glomerulonephritis was induced in Sprague Dawley rats (4–6 weeks old) by intravenous (i.v.) administration of 1 ml antithymocyte serum per 100 g body weight⁹. The anti-TGF-β1 serum and the two rabbit sera used as controls were also administered i.v. All sera were heat-inactivated at 56 °C for 30 min before injection. The extent of glomerular injury was evaluated by performing glomerular cell counts from 30 randomly selected glomeruli from 10 normal animals and nephritic animals in each group on days 4 and 7. Normal rat glomeruli contained 52 ± 14 cells. On day 4 there was a decreased number of cells (35 ± 11) as a result of cell lysis by the antithymocyte antibody, whereas an increased number of cells was seen on day 7 (68 ± 15). Values are mean $\pm$ s.d. The changes in cellularity were the same in the anti-TGF-β1 treatment and control groups.

against fibronectin, laminin and type IV collagen showed that the pathological matrix, as well as the normal matrix, contained each of these proteins (results not shown).

In a previous study we found that the injured glomeruli in culture produce more extracellular matrix components than do cells from normal glomeruli, and that the synthesis of two proteoglycans, biglycan and decorin, is particularly high⁹. This pattern of proteoglycan synthesis is similar to that obtained by adding TGF- β 1 to cultures of rat mesangial cells from normal glomeruli¹⁷, and antibody inhibition experiments and TGF- β 1 assays have shown that it is caused by increased synthesis of TGF- β 1 in the glomeruli⁹. We therefore used the production of proteoglycans as a bioassay that would reflect the activity of TGF- β and its influence on extracellular matrix synthesis in our disease model. Such an analysis showed that proteoglycan production by glomerular cells was suppressed to a near normal rate by anti-TGF- β 1 (Fig. 4a). Scans of the gel bands (Fig. 4a) and other similar experiments indicated that the suppression of this measure of the disease process was about 60% on day 4 and 80% on day 7 after injection. These results show that the glomerular disease was substantially attenuated by the anti-TGF- β 1 treatment.

The level of TGF- β 1 messenger RNA in the glomeruli of glomerulonephritis rats is higher than in normal rats and these glomeruli contain more cells producing TGF- β 1 than do normal glomeruli⁹. These results indicate that increased expression of the TGF- β 1 gene is correlated with injury in this model. Messenger RNA analysis of the anti-TGF- β 1-treated and control glomeruli on the current study revealed equally raised levels of TGF- β 1 mRNA in both groups of rats (Fig. 4b). A similar increase in the number of TGF- β 1-positive cells was also seen in the treated and control animals (results not shown). These results indicate that anti-TGF- β 1 did not interfere with induction of glomerular injury, as reflected by the increased synthesis of TGF- β 1 mRNA and TGF- β 1 protein in glomeruli. The cells responsible for the increased TGF- β 1 expression have not been identified, but they could be proliferating mesangial cells and/or infiltrating monocyte/macrophages.

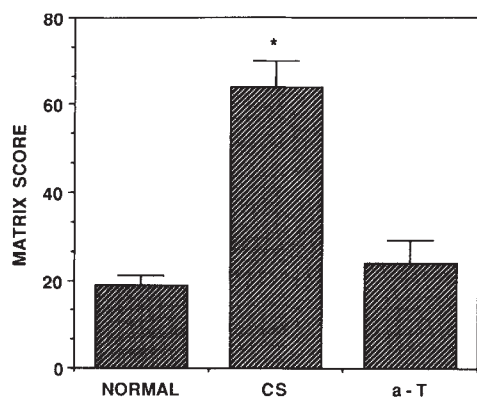


FIG. 3 Quantitation of extracellular matrix accumulation in nephritic glomeruli. Asterisk denotes $P < 0.001$, for glomerulonephritic rats treated with control serum (CS) compared with anti-TGF- β 1 (a-T) on day 7 of glomerulonephritis. Normal (normal) rats were included for comparison. Values are mean $\pm$ s.d. METHODS. To quantitate mesangial matrix all sections were coded and read by an observer unaware of the experimental protocol applied. Thirty glomeruli (80–100 μ m in diameter) were selected at random in sections prepared from normal rats or from anti-TGF- β 1 rats and control-treated rats on day 7 of glomerulonephritis. The degree of glomerular matrix expansion was determined as the percentage of each glomerulus occupied by mesangial matrix by using a published method³¹. Differences between groups in matrix scores were analysed by *t*-test. Two types of control sera were used: a normal rabbit serum, and a rabbit antiserum prepared against an unrelated peptide. Neither had any effect on the glomerulonephritis, and the results were pooled for the figure.

Our findings establish a central role for TGF- β in the pathogenesis of acute mesangial proliferative glomerulonephritis. TGF- β can greatly stimulate the production of extracellular matrix components by various kinds of cells^{10–16}, including the production of the two proteoglycans^{20,21} we have used as markers of TGF- β activity in this and earlier studies^{9,17}. Because of this activity, TGF- β has been suspected to have a role in fibrotic diseases resulting from various chronic disease processes^{22,23}. Whereas earlier results have provided correlative evidence for a role of TGF- β in glomerulonephritis, the antibody-inhibition data presented here establish that TGF- β has a direct, causal role, at least in the experimental glomerulonephritis model we have used. The type of TGF- β responsible for the increased production of extracellular matrix in our model

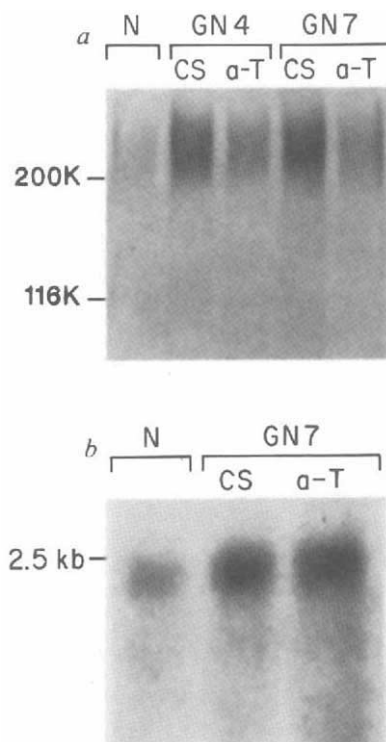


FIG. 4 a, Proteoglycan synthesis by glomeruli from glomerulonephritic rats treated with anti-TGF- β 1. CS, glomerulonephritic rats treated with control serum; a-T, glomerulonephritic rats treated with rabbit anti-TGF- β 1 on days 4 (GN 4) and 7 (GN 7) after injection of antithymocyte serum. The control lane (N) shows proteoglycan production in glomeruli from normal rat kidney. b, Northern blotting of TGF- β 1 mRNA in glomeruli isolated from the kidneys of normal and treated glomerulonephritic rats. Scanning of the bands showed a fivefold increase, relative to normal controls, in TGF- β 1 mRNA in both anti-TGF- β 1 (a-T) treatment and control (CS) groups on day 7. The control lane (N) shows TGF- β 1 mRNA in glomeruli from normal rat kidney. METHODS. a, Glomeruli were isolated 4 and 7 days after an antithymocyte serum injection that had been followed by treatments similar to those described in the legend of Fig. 3, and placed in culture⁹. Proteoglycan synthesis was examined by labelling the cultures with $^{35}\text{S}\text{O}_4$ followed by analysis of the secreted products by SDS-PAGE and autoradiography as described^{9,17}. Markers were myosin and β -galactosidase with relative molecular masses (M_r) of 200,000 and 116,000, respectively ($M_r \times 10^{-3}$). b, Total RNA was prepared by lysis of isolated glomeruli in guanidine isothiocyanate and ultracentrifugation of lysate on a caesium chloride cushion³³. Samples of 10 μ g were electrophoresed in a 2.2M formaldehyde/1% agarose gel and transferred to a nitrocellulose membrane. The membranes were prehybridized for 5 h at 37 $^\circ\text{C}$ in 5 $\times$ SSC, 5 $\times$ Denhardt's solution, 0.1 mg ml⁻¹ salmon sperm DNA, 0.1% SDS and 50% formamide. A porcine TGF- β 1 complementary DNA probe³⁴ and a rat glyceraldehyde 3-phosphate dehydrogenase cDNA probe³⁵ were labelled with [^{32}P]CTP by the random primer method and hybridized at 42 $^\circ\text{C}$ for 15 min. The TGF- β 1 mRNA result is shown; the amount of the dehydrogenase mRNA was equal in all samples and is not shown.

is likely to be TGF- β 1 because the antiserum was prepared against a peptide from this TGF- β . Because the amino-acid sequence of the various TGF- β s are similar^{14,24}, however, the antiserum could also have affected other types of TGF- β .

The mechanism of TGF- β 1 action is likely to be the extremely strong stimulation of extracellular matrix synthesis, manifested as a marked induction of proteoglycan synthesis by TGF- β 1 in cultural mesangial cells and of proteoglycan, fibronectin and other matrix molecules in cultured glomerular epithelial cells^{17,25}. The ability of TGF- β 1 to suppress the expression of proteases and stimulate the expression of protease inhibitors^{26,27} may also contribute to the accumulation of extracellular matrix.

We have used antithymocyte serum to produce acute mesangial matrix expansion *in vivo*. Although the aetiological mechanism in man is not known, accumulation of mesangial matrix is also a prominent and important pathological feature of human mesangial proliferative glomerulonephritis²⁸ and diabetic nephropathy³². The impressive suppression of the experimental disease achieved with anti-TGF- β 1 treatment indicates the importance of TGF- β in regulating extracellular matrix in glomerulonephritis. This suggests that TGF- β may have a similar role in human glomerular diseases, and perhaps other diseases in which fibrosis is a factor. $\square$

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Voltage-dependent InsP_3 -insensitive calcium channels in membranes of pancreatic endoplasmic reticulum vesicles

Andreas Schmid, Martine Dehlinger-Kremer, Irene Schulz & Heinz Gögelein

Max-Planck-Institut für Biophysik, Kennedy-Allee 70, D-6000 Frankfurt (Main) 70, FRG

STIMULUS-SECRETION coupling in exocrine glands involves Ca^{2+} release from intracellular stores^{1,2}. In endoplasmic reticulum vesicle preparations from rat exocrine pancreas, an inositol 1,4,5-trisphosphate (InsP_3)-sensitive, as well as an InsP_3 -insensitive, Ca^{2+} pool has been characterized³. But Ca^{2+} channels in the endoplasmic reticulum of rat exocrine pancreas have not been demonstrated at the level of single-channel current. We have now used the patch-clamp technique on endoplasmic reticulum vesicles fused by means of the dehydration-rehydration method^{4–6}. In excised patches, single Ba^{2+} - and Ca^{2+} -selective channels were recorded. The channel activity was markedly voltage-dependent. Caffeine increased channel open-state probability, whereas ruthenium red and Cd^{2+} blocked single-channel currents. Ryanodine, nifedipine and heparin had no effect on channel activity. The channel activity was not dependent on the free Ca^{2+} concentration, the presence of InsP_3 , or pH. We conclude that this calcium channel mediates Ca^{2+} release from an intracellular store through an InsP_3 -insensitive mechanism.

The endoplasmic reticulum (ER) of rat exocrine pancreas is known to play a central part in the regulation of cytoplasmic free Ca^{2+} concentration^{1,7,8}. To investigate whether Ca^{2+} channels are involved in the Ca^{2+} release mechanisms, isolated ER vesicles were fused to giant vesicles (10–100 μm) by the dehydration-rehydration method^{4–6}. These giant ER vesicles were used

for studies with the patch-clamp technique. To avoid current fluctuations from the large conductance anion channel⁵, Cl^- was replaced by HEPES buffer in both bath and pipette solutions. With K^+ buffer (75 mM KOH, 280 mM HEPES buffer, pH 7.1) in the pipette and Ba^{2+} buffer (50 mM $\text{Ba}(\text{OH})_2$, 280 mM HEPES buffer, pH 7.3) in the bath, single-channel currents carried by Ba^{2+} ions flowing from the bath into the pipette were recorded in 87% of stable seals. The mean channel conductance was 47 ± 3 pS ($n = 19$). Replacement of Ba^{2+} by Ca^{2+} showed that the channel is also permeable to Ca^{2+} ions. A single membrane patch contained 1–4 (usually 1 or 2) channels.

Typical single-channel current traces are shown in Fig. 1a. The conductance of this channel determined by linear regression was 41 pS (Fig. 1b). From the extrapolated reversal potential of -31 mV, a permeability ratio $P_{\text{Ba}^{2+}}/P_{\text{K}^+}$ of $\sim 15:1$ is estimated. The channel activity displayed a distinct voltage-dependence (Fig. 1a and c). Positive clamp voltages ($+10$ to $+20$ mV; sign refers to bath side) increased channel open-state probability, whereas negative potentials led to channel inactivation. In addition to the full current amplitude, sublevels of conductance occurred (Fig. 1a). Frequency, duration and magnitude of the sublevels varied in different experiments. As well as the voltage-dependent inactivation, in most experiments a spontaneous run-down of channel activity occurred with time; channel activity could be restored by applying short voltage pulses (± 40 mV).

To further characterize the channel, several potential Ca^{2+} channel activators and blockers were tested. Ryanodine^{9,10} (pipette 200 μM , $n = 11$; bath 50 μM , $n = 5$), which is known to arrest the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum (SR) on a sublevel of conductance, had no effect on Ca^{2+} channels in pancreatic ER membranes. Neither heparin (100 $\mu\text{g ml}^{-1}$, $n = 7$), an inhibitor, nor InsP_3 (10–50 μM , $n = 10$), an activator of InsP_3 -sensitive Ca^{2+} channels in smooth muscle SR¹¹, nor nifedipine (10–100 μM , $n = 9$), a blocker of voltage-dependent dihydropyridine-sensitive L-type calcium channels¹², had any detectable effects on either side of the membrane patch. The Ca^{2+} channel in fused pancreatic ER membranes, therefore,

clearly has different pharmacological properties from Ca^{2+} channels in other tissues.

In contrast to skeletal muscle SR calcium channels¹³, the activity of pancreatic ER calcium channels is independent of Ca^{2+} concentration. Thus, removal of Ca^{2+} ions from the pipette (5 mM EGTA; free $\text{Ca}^{2+} < 10^{-9}$ M; $n=9$) did not suppress channel activity, and neither elevated Ca^{2+} concentrations on the pipette side (free $\text{Ca}^{2+} 10^{-3}$ M; $n=19$), nor addition of Ca^{2+} ions (free $\text{Ca}^{2+} 10^{-5}$ M; $n > 20$) to the bath solution influenced channel appearance. Channel activity was also unaffected by

changes in pH on the pipette side from pH 6.7 (40 mM KOH, 320 mM HEPES buffer; $n=8$) to pH 7.8 (115 mM KOH, 180 mM HEPES buffer; $n=6$).

Caffeine (10 mM), which is known to activate cardiac SR Ca^{2+} channels¹⁴, also increased activity of Ca^{2+} channels in pancreatic ER membranes (Fig. 2b). This effect was, however, only observed in 4 out of 10 experiments. Ruthenium red (10 μM), an inhibitor of skeletal SR Ca^{2+} channels¹⁵ reduced Ca^{2+} channel activity (Fig. 2c).

The Ca^{2+} channels in ER membranes can also be blocked by

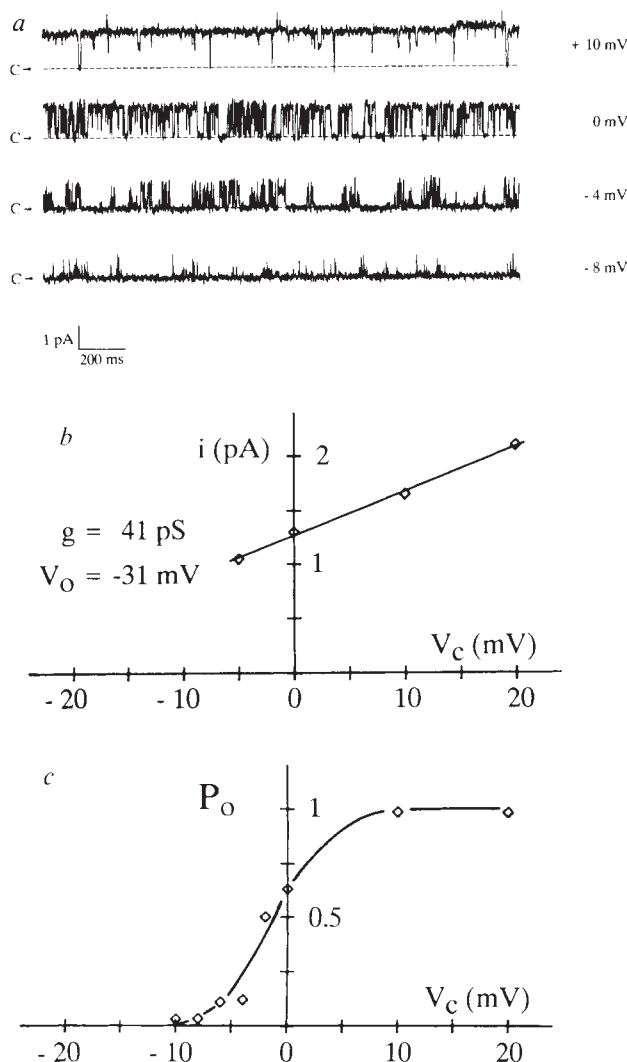
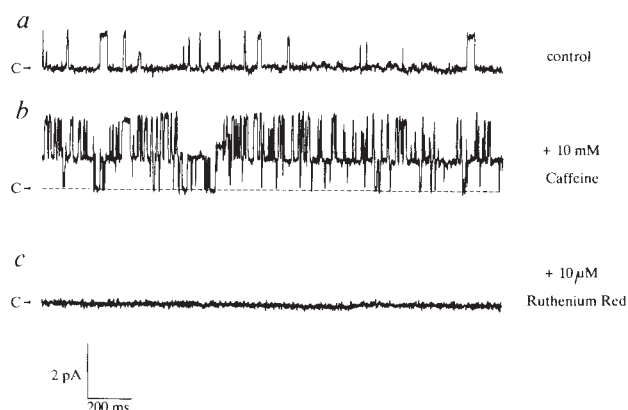


FIG. 1 a, Single-channel currents in a membrane patch excised from a giant vesicle produced by fusion of ER vesicles from rat pancreatic acinar cells. C→ marks the baseline current (closed state). Channel openings are shown by upward currents, indicating flow of Ba^{2+} ions from the bath into the pipette. The clamp potentials on the right refer to the bath. The channel activity is markedly voltage-dependent: at +10 mV the channel is open most of the time, but even small negative clamp potentials lead to drastic reduction of channel open times and increase of closed times. At -8 mV channel openings are only visible as short current spikes. The corresponding current-voltage data are shown in b. The data points are fitted to a straight line. Extrapolation to the voltage axis yields a reversal potential (E_{rev}) of -31 mV. c, The channel open state probability (P_O) versus the clamp potential (V_C). The curve was fitted by eye.

METHODS. ER vesicles were prepared from isolated rat exocrine pancreas cells²³. Cells were homogenized in 280 mM mannitol, 10 mM KCl, 5 mM HEPES buffer, 1 mM MgCl_2 , 1 mM benzamidine, pH 7.0 with Tris buffer. Cell debris, nuclei and mitochondria were removed from the homogenate by centrifugation at 400g and 11,000g (15 min). The resulting supernatant was centrifuged at 27,000g for 15 min, the pellet resuspended and adjusted to protein concentrations of 10–20 mg ml⁻¹. Vesicle fusion was performed with the dehydration–rehydration technique^{5,6}; droplets (10 μl) of the vesicle suspension were dehydrated in a desiccator by vacuum evaporation (10–20 min) and K^+ -HEPES buffer (75 mM KOH, 280 mM HEPES, pH 7.1) added for rehydration. Fusion to giant vesicles occurred within 0.5–2 h. Clusters of the still aggregated, rehydrating vesicles were transferred into the measuring chamber and allowed to settle for 10–30 min, then the K^+ -HEPES solution was carefully replaced by a Ba^{2+} -HEPES solution (50 mM $\text{Ba}(\text{OH})_2$, 280 mM HEPES, pH 7.3). Measurements were carried out at room temperature (20–22 °C). The tip of the patch pipette was filled with K^+ -HEPES buffer overlain with 140 mM KCl solution. The bath was connected by an agar bridge to a Ag/AgCl electrode in 140 mM KCl. Clamp voltages were corrected for the reversal potential of the respective baseline current, which in most experiments was within the range 0 to +5 mV. $P_{\text{Ba}^{2+}}/P_{\text{K}^+}$ was calculated according to the constant field theory: $P_{\text{Ba}^{2+}}/P_{\text{K}^+} = a_K \cdot (\exp^u + 1)/4 \cdot a_{\text{Ba}} \cdot \exp^{2u}$, where $u = F \cdot E_{rev}/R \cdot T$, and assuming activity coefficients of about 0.78 for γ_{K^+} and about 0.29 for $\gamma_{\text{Ba}^{2+}}$ ²⁴. Further details about patch-clamp recording technique and data analysis were described previously²⁵.

FIG. 2 Activity of Ca^{2+} channels in membranes of fused ER vesicles can be increased by caffeine. C→ marks the closed state. Channel openings are shown by upward currents. The clamp potential was -10 mV. Under control conditions channel openings occurred in short sporadic spikes (a). Sixty seconds after addition of caffeine (10 mM) to the bath, activity of two channels with increased open probability was observed (b). A similar increase in channel activity was never observed under control conditions. Addition of 10 μM ruthenium red to the bath solution led to complete inactivation of the Ca^{2+} channels (c). Channel activity could be restored by bath perfusion with control solution. In some experiments, when ruthenium red was added to the bath solution, only incomplete channel blockage was observed. The blocking effect was more pronounced when ruthenium red was applied from the pipette side. As fluid exchange in the pipette was not feasible because of poor stability of the membrane seals, different membrane patches have to be compared. Under control conditions, in 87% of stable seals ($n=60$) Ca^{2+} channels were detectable. With 10 μM ruthenium red in the pipette solution single calcium channels retained activity in only 2 out of 20 experiments. In all other cases channel activity was still observed during the first 1–2 seconds after seal formation but then disappeared completely.



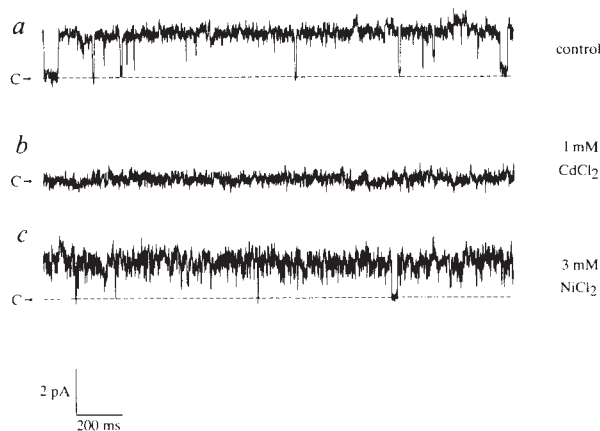


FIG. 3 Effects of Cd^{2+} and Ni^{2+} on Ca^{2+} channel activity in pancreatic ER membranes. *a*, Normal channel activity under control conditions. V_c was +5 mV. *b*, Addition of 1 mM Cd^{2+} to the bath solution resulted in rapid and complete channel blockage. In contrast, 3 mM Ni^{2+} did not suppress channel activity but induced rapid flickering with decrease in mean current amplitude (*c*). The effects of both Cd^{2+} and Ni^{2+} were fully reversible.

addition of Cd^{2+} to the bath (Fig. 3*b*), as observed for Ca^{2+} channels in heart ventricular cells¹⁶. Complete channel blockage was achieved by application of 500 μM Cd^{2+} (0.5–1 mM; $n=9$). Ni^{2+} was a less potent inhibitor (Fig. 3*c*); millimolar concentrations (1–3 mM) induced rapid flickering but did not suppress channel activity ($n=7$). These effects were fully reversible.

The voltage-dependent Ca^{2+} channels described in this study were neither dependent on the presence of InsP_3 , nor was channel activity suppressed by heparin. We therefore conclude that these channels are not involved in the InsP_3 -triggered Ca^{2+} release mechanism in pancreatic ER membranes. However, we have never observed InsP_3 -induced Ca^{2+} channels, as had been found in smooth muscle SR vesicles¹¹; InsP_3 -induced Ca^{2+} channels in the ER of the exocrine pancreas of the rat could be too small to be detected (<5 pS).

Recent studies of several cell types provide evidence for the existence of more than one non-mitochondrial intracellular Ca^{2+} store and Ca^{2+} release mechanism^{3,17–20}. For example, Ca^{2+} -induced Ca^{2+} release similar to that in skeletal muscle SR was implicated in rat lacrimal gland cells¹⁷. However, the Ca^{2+} channels found in rat exocrine pancreas ER membranes are clearly different from SR Ca^{2+} channels. The conductance is smaller, a ryanodine effect could not be observed and calcium on the pipette side—which is assumed to represent the cytosolic membrane side—has no activating effect. Thus, we conclude that the voltage-dependent Ca^{2+} channels described in this study are not identical to the Ca^{2+} release mechanism observed in SR membranes. Studies on Ca^{2+} uptake into ER vesicles from rat exocrine pancreas revealed at least two distinct Ca^{2+} pools, an InsP_3 -sensitive and an InsP_3 -insensitive one³. Further studies showed that Ca^{2+} ions can be released from an InsP_3 -insensitive pool by caffeine (M.D.-K. and I.S., manuscript in preparation). The type of voltage-dependent Ca^{2+} channel described in this study is probably located in this caffeine-sensitive Ca^{2+} pool. The most striking feature of the Ca^{2+} channel in ER membranes is its voltage-dependence, which means that Ca^{2+} release from the vesicles would be inhibited by inside negative voltages. Current models for function of InsP_3 -sensitive and InsP_3 -insensitive Ca^{2+} pools involve GTP-induced connection between different Ca^{2+} pools^{21,22}. One possible physiological signal inducing Ca^{2+} release through voltage-dependent Ca^{2+} channels could therefore be linkage of Ca^{2+} channel-containing vesicles to other vesicles that possess either an inside positive potential or ion permeabilities short-circuiting the membrane potential. □

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Escape of DNA from mitochondria to the nucleus in *Saccharomyces cerevisiae*

Peter E. Thorsness & Thomas D. Fox

Section of Genetics and Development, Cornell University, Ithaca, New York 14853-2703, USA

THE migration of genetic information from ancestral prokaryotic endosymbionts into eukaryotic nuclei is thought to have had an important role in the evolution of mitochondria and chloroplasts^{1–6}. Here we describe an assay for the detection of movement of DNA between mitochondria and the nucleus in yeast. Because recombinant plasmid DNA replicates after transformation into mitochondria of yeast strains lacking endogenous mitochondrial DNA⁷ we were able to propagate the nuclear genetic marker *URA3* in mitochondria. As expected, the wild-type *URA3* gene in mitochondria failed to complement the uracil auxotrophy (*Ura*[−]) caused by a nuclear *ura3* mutation. But selection of *Ura*⁺ prototrophs from a *Ura*[−] strain carrying *URA3* on a plasmid in its mitochondria enabled us to detect plasmid movement to the nucleus. Conversely, as the plasmid used also contained the mitochondrial gene *COX2* required for respiratory growth, we were able to set up corresponding selections to detect migration of DNA from the nucleus to the mitochondria. Our results show that, in yeast, DNA escapes from mitochondria and appears in the nucleus at a surprisingly high frequency ($\sim 2 \times 10^{-5}$ per cell per generation). But the rate at which DNA makes the journey in the opposite direction—nucleus to mitochondria—is apparently at least 100,000 times less.

A yeast strain with mitochondria that contained only sequences derived from a defined plasmid was generated using high velocity microprojectile bombardment^{7–9}. If a ρ^0 yeast strain, lacking endogenous mitochondrial DNA (mtDNA), is bombarded with a plasmid carrying the mitochondrial gene *COX2* (previously referred to as *oxi1* (refs 10, 11)), rare transformants can be isolated in which the plasmid is present in mitochondria⁷. These transformants (synthetic ρ^- strains) mimic the behaviour of natural ρ^- strains, which lack a mitochondrial translation system and thus fail to respire. But as the transformants contain many copies of the *COX2* gene in mitochondria they can, like natural ρ^- strains retaining *COX2*, complement the respiratory defect of a ρ^+ strain (that retains mitochondrial translation) with a *cox2* deletion. The ability to complement is evidence that

TABLE 1 Yeast strains

Strain	Nuclear genotype	Mitochondrial genotype
TF189 ρ^+	<i>MATa, ade2-101, ura3-52</i>	ρ^+
TF189 ρ^0	<i>MATa, ade2-101, ura3-52</i>	ρ^0
PTY1	<i>MATa, ade2-101, ura3-52, [URA3 (pMK2)]</i>	ρ^- , <i>COX2</i> (pMK2)
PTY2	<i>MATa, ade2-101, ura3-52</i>	ρ^- , <i>COX2</i> , (pMK2)
PTY3	<i>MATa, ade2-101, ura3-52, [URA3 (pMK2)]</i>	ρ^0
PTY6	<i>MATa, ade2, ura3-52</i>	ρ^+ , <i>cox2-17</i>
PTY16	<i>MATa, ino1, can', ura3-52</i>	ρ^- , <i>COX2</i> (pMK2)
PTY17	<i>MATa, trp1-Δ1, ura3-52</i>	ρ^- , <i>COX2</i> (pMK2)
PARB1	<i>MATa, ade2-101, ura3-52</i>	ρ^+ , <i>cox2-17</i>

Yeast strains TF189 ρ^+ , TF189 ρ^0 , PTY1, PTY2, PTY3 and PARB1 are all isogenic to DBY947 (ref. 25). PARB1 was obtained from P. Anziano and R. A. Butow. PTY16 was derived from strain TD28 ρ^0 (ref. 7). PTY17 was derived from D273-10B (ATCC25657). The *cox2-17* allele of PTY6 is a complete deletion of the *COX2* coding region of mtDNA, previously termed *oxi1-17* (ref. 7). Strain PTY1 was made by bombardment of TF189 ρ^0 with pMK2-coated tungsten particles essentially as described⁷. pMK2 DNA (17 μ g) were precipitated on to 3 mg of 1.2- μ m tungsten particles in the presence of 1.25 M CaCl₂ and 10 mM spermidine. One fifth (0.6 mg) of these DNA-coated tungsten particles were used to bombard a minimal glucose-adenine agar plate (lacking uracil) spread with about 1×10^8 cells. After bombardment of 40 plates, ~2,000 nuclear transformants (Ura⁺ cells) were screened for the presence of pMK2 DNA in the mitochondria by mating with a ρ^+ , *cox2-17* tester and subsequent screening for respiring diploids. PTY3 was a nuclear transformant picked from a bombarded lawn that failed to complement the ρ^+ , *cox2-17* tester. PTY2 was a mitotic segregant of PTY1 that had lost pMK2 from the nucleus. PTY16 and PTY17 were constructed by transfer of ρ^- [pMK2] mitochondria into ρ^0 strains with the corresponding nuclear genetic backgrounds. The mitochondria were passaged through a ρ^0 derivative of the *kar1-1* yeast strain, 8960-11B (obtained from G. R. Fink).

the transformants contain the plasmid in mitochondria because, as noted previously⁷ and confirmed below, mitochondrial genes on plasmids present in the yeast nucleus are genetically inactive.

Our experiment required a recombinant plasmid whose presence could be detected in either the nuclear or mitochondrial compartments. The plasmid pMK2 contains the *URA3* gene¹², a common nutritional marker for nuclear transformation in yeast, and the mitochondrial marker *COX2* which encodes subunit II of cytochrome oxidase. In addition, pMK2 contains the origin of replication of the yeast 2 μ plasmid, allowing autonomous propagation in the nucleus. There is apparently no

specific sequence required for replication of DNA in the mitochondria of ρ^- yeast¹³.

The yeast strain TF189 ρ^0 (Table 1), a uracil auxotroph by virtue of the nonreverting *ura3-52* allele¹⁴, was bombarded by particles coated with pMK2 DNA⁷. One strain recovered, designated PTY1 (Table 1), had pMK2 DNA in its mitochondria, judged on the basis of its ability to complement a *cox2* deletion mutant after mating. A Ura⁻ mitotic segregant of PTY1, named PTY2, was isolated and shown to contain pMK2 sequences, unrearranged except for the expected presence of a high proportion of concatemers characteristic of ρ^- mtDNA^{7,15} (Fig. 1). PTY2, like many natural ρ^- strains¹⁵, also contained monomer circles. PTY2 was auxotrophic for uracil despite the fact that it contained a *URA3* gene, because that gene was located in mitochondria, not in the nucleus. (As a ρ^- strain lacking mitochondrial protein synthesis, PTY2 cannot express *URA3*.) Figure 2a is a schematic depiction of PTY2, a strain with copies of a nuclear gene located in mitochondria that are potentially capable of complementing a nuclear mutation.

Several clones of PTY2 were grown on complete medium agar plates at 30 °C. Individual clones were excised, suspended in water and transferred to agar plates lacking uracil in order to select cells that had become Ura⁺ despite carrying the nonreverting nuclear *ura3-52* mutation. Numerous Ura⁺ colonies were observed from every ρ^- clone. Using the statistical analysis of Luria and Delbrück¹⁶ we found that PTY2 yielded Ura⁺ prototrophs at a rate of $\sim 2 \times 10^{-5}$ per cell generation under these conditions (Table 2).

The appearance of Ura⁺ prototrophs depended strictly on the presence of pMK2 DNA in the mitochondria. More than 50 mitotic segregants of PTY2 were isolated that no longer contained pMK2 DNA in their mitochondria, as judged by their inability to complement a *cox2* deletion. In every case, these segregants failed to give rise to Ura⁺ prototrophs. This strong linkage between *COX2* and *URA3* confirmed that the source of genetic information allowing complementation of the *ura3-52* mutation in PTY2 was pMK2 DNA contained within the mitochondria.

The Ura⁺ derivatives selected from PTY2 behaved as though pMK2 was replicating as a plasmid in their nuclei. First, the Ura⁺ phenotype was mitotically unstable, as expected from a 2 μ -based plasmid¹⁷. Second, DNA was isolated from several Ura⁺ prototrophs, as well as from strains derived from these prototrophs that had lost pMK2 from the mitochondria. Gel-blot hybridization analysis revealed that the pMK2 plasmid sequences transferred to the nucleus had not been rearranged during

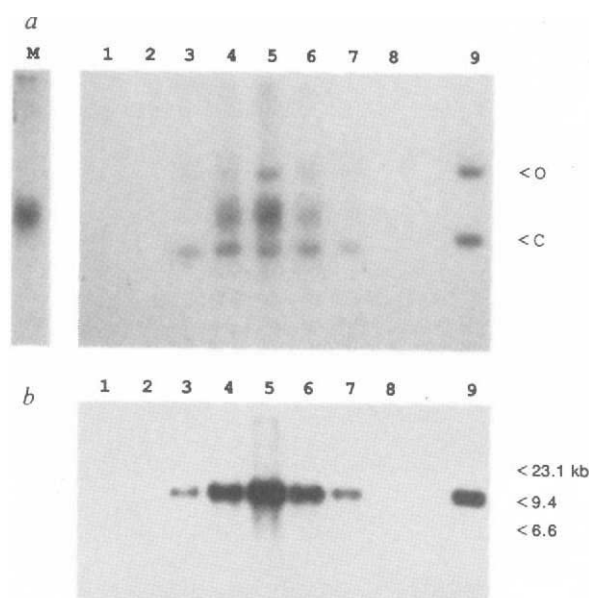


FIG. 1 Gel-blot-hybridization analysis of DNA from yeast strains containing pMK2 in their nuclei and/or mitochondria. a, Undigested DNAs: Total cellular DNA from strains TF189 ρ^+ (lane 1), TF189 ρ^0 (lane 2), PTY3 (lane 3), PTY1 (lane 4), PTY2 (lane 5), a Ura⁺, ρ^- derivative of PTY2 (lane 6), a Ura⁺, ρ^0 derivative of PTY2 (lane 7), a Ura⁻, ρ^0 derivative of PTY2 (lane 8) and CsCl gradient purified pMK2 (lane 9) were run on an agarose gel and blotted to a filter. Samples in lanes 1-9 were probed with ³²P-labelled pBR322 DNA (the backbone of plasmid pMK2) and autoradiographed. The positions of open (O) and closed (C) circular pMK2 are indicated. In lane M, DNA from TF189 ρ^+ was run and probed with yeast [³²P] mtDNA purified on a CsCl gradient. The broad band of mtDNA in lane M is at the same position as the signals corresponding to multimeric plasmid DNA in strains containing pMK2 in their mitochondria (lanes 4, 5 and 6). b, DNAs digested with EcoRI before electrophoresis and the filter probed with ³²P-labelled pMK2 DNA. In every case, plasmid DNA was reduced to the same size as purified pMK2 (lane 9). DNA isolation, electrophoresis in 1% agarose containing Tris-phosphate buffer with 1 μ g ml⁻¹ ethidium bromide, blotting and hybridization were carried out as previously described²⁶⁻²⁸. Abbreviation: kb, kilobases.

TABLE 2 Rate of appearance of uracil prototrophs in strains with pMK2 in their mitochondria

Strain	Growth temperature (°C)	Events per cell per generation
PTY2	30	2×10^{-5}
PTY16	30	9×10^{-5}
PTY17	30	4×10^{-5}
PTY17	37	10×10^{-5}
PTY17	14	8×10^{-5}

Cells grown in complete medium were either micromanipulated or diluted and spread on to complete-medium agar plates to obtain colonies derived from single cells. Between 10 and 20 independent colonies were examined for each strain. After growth for several days at the indicated temperature, whole colonies (about 5×10^6 cells) were excised and resuspended in 1 ml sterile water. The total cells per colony and Ura⁺ cells per colony were determined by plating aliquots on complete medium, and medium lacking uracil, respectively. In each case, the rate at which uracil prototrophs arose was determined numerically using the equation derived by Luria and Delbrück¹⁶, $r = aN_t \ln(N_t/Ca)$ where r is the observed average number of Ura⁺ cells per colony, C is the number of colonies examined, N_t is the total number of cells in a colony, and a is the number of Ura⁺ events per time unit. The rates reported above are the value of a multiplied by $\ln 2$.

their propagation or migration (Fig. 1). Indeed, the DNA from the Ura⁺ prototrophs derived from PTY2 by escape of pMK2 from mitochondria to the nucleus was indistinguishable from that of the original transformant PTY1.

These results strongly support the notion that a cytological event allows pMK2 to escape from mitochondria and enter the nucleus (Fig 2a, c), and that this event does not involve a genetic mutation. As further confirmation of this idea, we repeated the entire series of experiments in cycles starting with Ura⁻ mitotic segregants selected from the Ura⁺ derivatives of PTY2. We were able repeatedly to isolate spontaneous Ura⁺ derivatives from Ura⁻ mitotic segregants, and the process remained strictly dependent on the maintenance of pMK2 sequences in mitochondria.

To test whether the frequency of pMK2 escape from mitochondria would vary in strains with different nuclear genetic backgrounds, the ρ^- mitochondria of strain PTY2 were transferred to ρ^0 derivatives of two other yeast strains, using a *kar1* mutant¹⁸ (Table 1). (The ability to complement *cox2* remained

completely linked to the ability to become Ura⁺ during these transfers, further supporting the notion that *URA3* was located in mitochondria.) The rate of pMK2 escape from mitochondria of PTY17 was twofold greater, and of PTY16 more than fourfold greater, than that of PTY2 (Table 2), indicating that genetic background affects the process that allows DNA to escape from mitochondria and migrate to the nucleus.

The rate of escape was also affected by temperature and the composition of the medium. Cells grown at either low (14 °C) or high (37 °C) temperature yielded more Ura⁺ segregants than cells grown under optimal conditions (30 °C) (Table 2). Furthermore, when cultures of PTY17 were frozen at -70 °C in water containing 15% glycerol, thawed and replated, the number of Ura⁺ derivatives increased threefold over that obtained by plating an unfrozen aliquot of the culture incubated at room temperature in glycerol for the same brief period. Incubation of PTY17 in 15% glycerol at 30 °C for 6 hours, however, resulted in a threefold increase in the proportion of Ura⁺ cells when compared with cells from the same culture incubated in water. (Incubation in water had no effect on the number of Ura⁺ cells in the sample.)

Escape of DNA from mitochondria occurs not only in respiratorily defective ρ^- cells, but also in respiring ρ^+ cells. We showed this by selecting respiring Ura⁺ diploids from Ura⁻ zygotic clones, generated by mating PTY2 with PTY6. The recovery of respiring cells, carrying *URA3* on a plasmid in the nucleus and expressing the plasmid-derived *COX2* gene in mitochondria (not shown), demonstrated escape in respiring cells. These diploids were heteroplasmic for two forms of mtDNA, and no calculation of the rate of escape could be made owing to the instability of the heteroplasmic state.

In light of the evidence demonstrating the migration of DNA from mitochondria to the nucleus at high frequency, we examined the possibility that movement might also occur in the reverse direction. We isolated a Ura⁺-transformed strain, PTY3 (Table 1), that contained the plasmid pMK2 only in the nucleus (shown schematically in Fig. 2b). If pMK2 could migrate from the nucleus into the mitochondria of this ρ^0 strain, then PTY3 would be expected to give rise to ρ^- derivatives (shown schematically in Fig. 2c) capable of complementing the *cox2* deletion of the ρ^+ strain PTY6 (Table 1). PTY3 was grown selectively to maintain the plasmid and $\sim 10^8$ cells were mated with an equal number of PTY6 cells. None of the resulting diploids (at least 5×10^7) gave rise to respiring colonies. So,

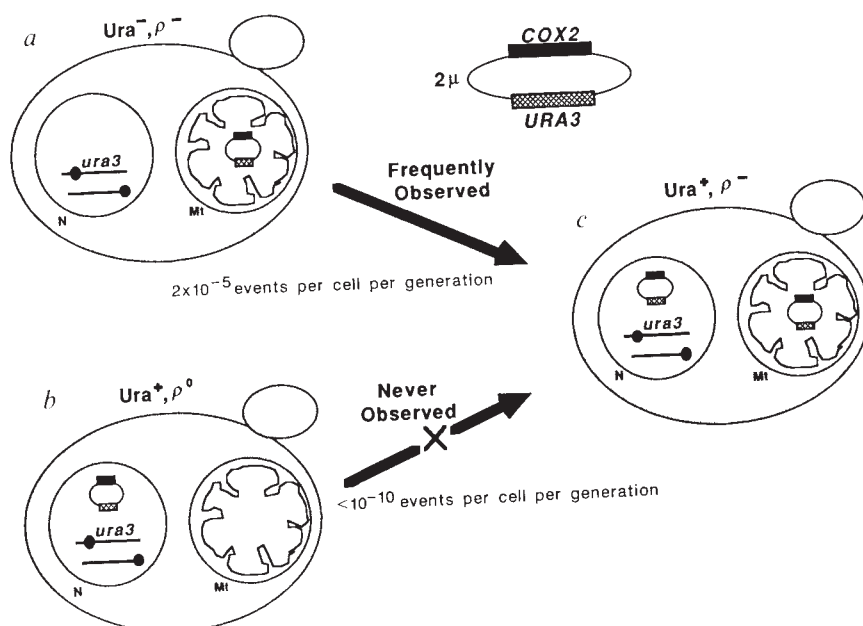


FIG. 2 Experimental scheme. A plasmid carrying the nuclear gene *URA3*, the 2 μ origin of replication and the mitochondrial gene *COX2* (pMK2) could be maintained in either the nucleus (N), the mitochondrion (Mt) or both compartments. a, Cells containing the plasmid in their mitochondria (equivalent to the yeast strain PTY2 (Table 1)) frequently give rise to cells containing the plasmid in both the nucleus and mitochondria, c. b, Cells containing the plasmid in the nucleus (equivalent to the yeast strain PTY3 (Table 1)) failed to give rise to cells with the plasmid in both compartments, c, at detectable frequency. As indicated, all strains carried a nonreverting chromosomal *ura3* mutation.

under these conditions, the movement of DNA from the nucleus to the mitochondria was at least 100 times less frequent than the observed movement in the opposite direction.

To select more strongly for movement from the nucleus to mitochondria, we directly transformed the nucleus of strain PARB1, a *ura3-52*, ρ^+ strain carrying the *cox2* deletion mutation (Table 1), with pMK2. The only means by which this nonrespiring transformant could give rise to respiring cells would be by the transfer of pMK2 sequences that contained the *COX2* gene from the nucleus to mitochondria. About 10^{10} cells were grown from this transformant under conditions selecting for maintenance of pMK2 in the nucleus and plated on complete media that required respiration for growth. No respiring colonies were observed. Thus, under conditions that allowed frequent transfer of DNA from mitochondria to the nucleus, no transfer of DNA from the nucleus to mitochondria was detected.

The mechanism by which DNA moves from mitochondria to nuclei is unknown. Uptake of DNA from the cytoplasm into nuclei seems to be a very general phenomenon. Yeast nuclei can be transformed by a variety of procedures^{7,9,19-22} that presumably introduce DNA from outside the cell into the cytoplasm. The nuclei can take up DNA molecules ranging in size from small plasmids to chromosome-sized pieces²³. Indeed, internuclear transfer of chromosomes has been observed in yeast heterokaryons²⁴. So how does DNA escape from mitochondria to the cytoplasm? One possibility is that transient breaches in mitochondrial membranes allow the release of mtDNA from the matrix. The increase in DNA migration observed in cells grown at high or low temperature, subjected to freezing or incubation with glycerol—actions that might disrupt mitochondrial membranes—is consistent with this hypothesis. Another possibility is that the escape events observed in these studies were initiated during terminal degradation of organellar structures that occasionally released intact DNA molecules. This hypothesis would explain the failure to observe plasmid migration into mitochondria. In any event, the assay described here for the movement of DNA within a eukaryotic cells makes possible further physiological and genetic studies that may define molecular mechanisms important in compartmentalization and organization of nuclei and mitochondria. □

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Triggering of cyclin degradation in interphase extracts of amphibian eggs by *cdc2* kinase

Marie-Anne Félix*, Jean-Claude Labbé†, Marcel Dorée‡, Tim Hunt‡ & Eric Karsenti*§

* EMBL, Postfach 102209, 6900 Heidelberg, FRG

† CNRS, BP 5051, 34033 Montpellier Cedex, France

‡ University of Cambridge, Department of Biochemistry, Tennis Court Road, Cambridge CB2 1QW, UK

THE cell cycles of early *Xenopus* embryos consist of a rapid succession of alternating S and M phases¹. These cycles are controlled by the activity of a protein kinase complex (*cdc2* kinase) which contains two subunits. One subunit is encoded by the frog homologue of the fission yeast *cdc2⁺* gene, p34^{*cdc2*} (ref. 2) and the other is a cyclin³. The concentration of cyclins follows a sawtooth oscillation because they accumulate in interphase and are destroyed abruptly during mitosis³. The association of cyclin and p34^{*cdc2*} (refs 4-7) is not sufficient for activation of *cdc2* kinase, however; dephosphorylation of key tyrosine and threonine residues of p34^{*cdc2*} is necessary to turn on its kinase activity⁸⁻¹¹. The activity of *cdc2* kinase is thus regulated by a combination of translational and post-translational mechanisms. The loss of *cdc2* kinase activity at the end of mitosis depends on the destruction of the cyclin subunits^{3,4,12,13}. It has been suggested that this destruction is induced by *cdc2* kinase itself, thereby providing a negative feedback loop to terminate mitosis^{14,15}. Here we report direct experimental evidence for this idea by showing that cyclin proteolysis can be triggered by adding *cdc2* kinase to a cell-free extract of interphase *Xenopus* eggs.

To study the control of cyclin degradation, [³⁵S]methionine-labelled sea urchin cyclin was made in a reticulocyte lysate programmed with synthetic messenger RNA¹⁶. A portion of the synthesis mix was added to an interphase extract prepared from activated *Xenopus* eggs¹⁵. Cyclin stability was monitored by gel electrophoresis and autoradiography and quantified by scanning densitometry. Labelled cyclin was reasonably stable in interphase extracts, but when 12 U μ l⁻¹ of active *cdc2* kinase was added, cyclin was rapidly destroyed after a 25-min lag (Fig. 1a). Raising the level of *cdc2* kinase to 24 U μ l⁻¹ reduced this lag to about 15 min, but did not increase the rate of destruction once it had started. Further increase in the level of *cdc2* kinase did not markedly shorten the lag phase or affect the kinetics of cyclin destruction. These results suggest that *cdc2* kinase initiates a chain of events, culminating in cyclin destruction. It is noteworthy that the minimum level of *cdc2* kinase required to trigger cyclin destruction in these extracts (12 U μ l⁻¹) is close to what is found during mitosis *in vivo*¹⁷ and in M-phase extracts¹⁵. The induction of cyclin proteolysis by *cdc2* kinase required the presence of *Xenopus* extract. It did not occur when the extract was replaced with buffer (Fig. 1a), nor when it was replaced by the rabbit reticulocyte lysate in which the cyclin had been synthesized (data not shown). When the *Xenopus* extract was diluted two- or fourfold, the rate of cyclin destruction was reduced but the length of the lag was essentially unaffected. A 10-fold-diluted extract did not support cyclin destruction (Fig. 1a).

These experiments used preparations of *cdc2* kinase purified by p13^{*suc1*} affinity chromatography as an equimolar complex of p34^{*cdc2*} and cyclin⁶ (Fig. 1b, lane 1). It was therefore possible that the proteolytic pathway was activated by the added cyclin component, and not by p34^{*cdc2*}. To examine this possibility, we repeated the experiments using a preparation of *cdc2* kinase lacking intact cyclin subunits¹⁸ (Fig. 1b, lane 2). This form of

§ To whom correspondence should be addressed.

the enzyme induced cyclin degradation as well as did cdc2 kinase with intact cyclin, and the same level of kinase activity was required (Fig. 1c). Therefore, it is unlikely that a threshold level of cyclin *per se* is the trigger for proteolysis.

It was possible that the added cdc2 kinase promoted cyclin degradation by activating the latent endogenous cdc2 kinase activity of the extract¹⁹. This did not seem to be the case, however, because during these incubations, there was no increase in the level of histone H1 kinase activity over and above the level attributable to the added cdc2 kinase. Indeed, an abrupt loss of kinase activity occurred concomitant with cyclin degradation (Fig. 2a). So addition of exogenous cdc2 kinase did not

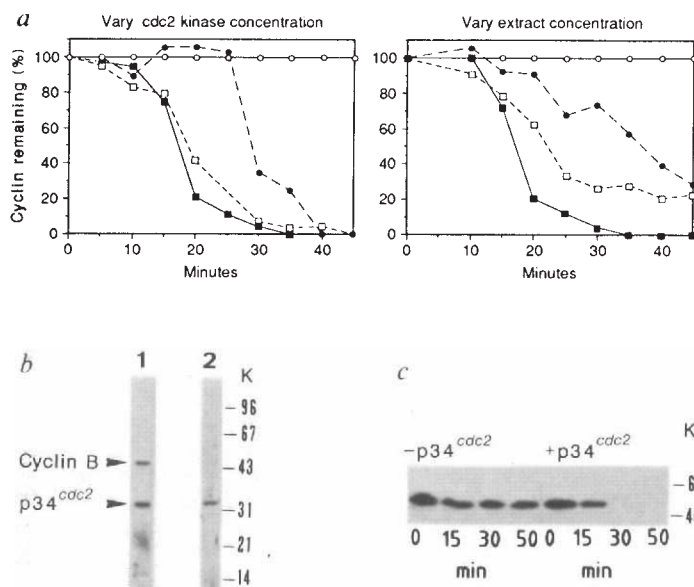


FIG. 1 Cyclin degradation in interphase extracts can be triggered by added cdc2 kinase. **a**, Left panel—effect of the level of cdc2 kinase activity on cyclin degradation. Kinase (prepared from starfish oocytes⁹) was added to give the following units of activity in the extracts: less than $6 \text{ U } \mu\text{l}^{-1}$ ($\circ$); $12 \text{ U } \mu\text{l}^{-1}$ ($\bullet$); $24 \text{ U } \mu\text{l}^{-1}$ ($\square$); $48 \text{ U } \mu\text{l}^{-1}$ ($\blacksquare$). The level of cyclin is expressed as a fraction of the amount present at each time point without added kinase. Right panel—effect of dilution of the *Xenopus* extract on cyclin degradation. The starting extract ($32 \text{ mg per ml protein}$) was the same as shown in the left panel. The extract was diluted twofold ($\square$), fourfold ($\bullet$) or 10-fold ($\circ$). For each time-point, the remaining cyclin is expressed as a fraction of the cyclin level in a parallel reaction with acetate buffer. All reactions contained $24 \text{ U } \mu\text{l}^{-1}$ cdc2 kinase, 1 mM Mg^{2+} -ATP, $10 \text{ mM creatine kinase}$ and $80 \text{ } \mu\text{g ml}^{-1}$ creatine phosphate. **b**, Silver-stained SDS-PAGE analysis of the two cdc2 kinase preparations. Lane 1, p34^{cdc2} associated with cyclin B (prepared as in ref. 16). Lane 2, homogeneous p34^{cdc2} catalytic subunit (prepared as in refs 18 and 28). Relative molecular mass (M_r) markers shown on the right ($M_r \times 10^{-3}$). **c**, Cyclin degradation induced by the addition of the p34^{cdc2} catalytic subunit. The cdc2 kinase shown in **b**, lane 2 ($20 \text{ U } \mu\text{l}^{-1}$) was added to the *Xenopus* extract together with ^{35}S -labelled cyclin. Cyclin degradation was monitored by SDS-PAGE and autoradiography. M_r markers, right ($M_r \times 10^{-3}$).

METHODS. *Xenopus* eggs were activated by electric shock and extracts prepared 40 min later by centrifugation at $100,000g$ for 1 h as previously described¹⁵. The $100,000g$ supernatants were frozen in liquid nitrogen. A full-length complementary DNA clone of sea urchin (*Arbacia punctulata*) cyclin was transcribed and the RNA translated in reticulocyte lysate in the presence of $7.5 \text{ } \mu\text{Ci } \mu\text{l}^{-1}$ [^{35}S]methionine as described previously¹⁶. The [^{35}S]cyclin and cdc2 kinases were added in less than 10% of the final incubation volume. One unit of kinase activity is defined as 1 pmol phosphate transferred from ATP to histone H1 per min. Cycloheximide ($10 \text{ } \mu\text{g ml}^{-1}$) was added to block further protein synthesis. At the indicated times of incubation at 22°C , samples were diluted sixfold into SDS-gel sample buffer and analysed on 10% polyacrylamide gels. The gels were exposed to preflashed X-AR film (Kodak) and the cyclin bands quantified on an LKB ultrascan XL laser densitometer. To measure cdc2 kinase activity in the reactions, samples were diluted 26-fold into 'extraction buffer' (EB, $80 \text{ mM } \beta$ -glycerophosphate, 15 mM MgCl_2 , 20 mM EGTA , 1 mM DTT , pH 7.3) and assayed for H1 kinase activity with calf thymus histones²⁸.

lead to detectable activation of the endogenous cdc2 kinase activity in these crude extracts. This contrasts with the findings of Dunphy and Newport¹⁹ who showed that the activity of maturation-promoting factor (MPF), assayed by the ability of MPF to trigger nuclear envelope breakdown in extracts of interphase *Xenopus* eggs, could be amplified in ammonium sulphate fractions of similar extracts. The two approaches differ in significant ways, however. We added highly purified cdc2 kinase to total cell extracts, whereas they used partially purified MPF added to an ammonium sulphate fraction of similar extracts. Furthermore they included ATP- γ -S in the incubation, which we did not. Finally, and perhaps most significantly, they were measuring MPF and not H1 kinase activity.

We conclude that cdc2 kinase promotes the destruction of cyclin by initiating a cascade of reactions that begins with protein phosphorylation and ends with proteolysis. The cascade, which may contain several steps, seems to have a built-in time delay corresponding roughly to the normal length of mitosis. The protein kinase activity of cdc2 kinase presumably triggers the cascade; we do not intend to imply that cdc2 kinase contains a cryptic protease, or even that the putative protease is directly activated by cdc2-mediated phosphorylation.

To test the idea that the kinase activity of the added p34^{cdc2} triggered proteolysis, we added 6-dimethylaminopurine (DMAP) to the reactions. This ATP analogue inhibits the histone H1 kinase activity of cdc2 kinase^{15,20}. Figure 2a shows that 1 mM DMAP substantially, although not completely, inhibited cyclin destruction; it also partially protected cdc2 kinase from inactivation. The activity of the kinase can still be measured under these conditions because the DMAP is diluted below its active concentration in the histone kinase assay. This interpretation is subject to the reservation that DMAP is an adenine analogue and might inhibit ATP-dependent reactions other than protein phosphorylation. To strengthen the case, we tested a more specific inhibitor of cdc2 kinase, the histone H1-like peptide KTPKKAKKPKTPKKAKKL (single-letter amino-acid code; KTPK peptide) which contains a repeat of the phosphorylation site for growth-associated H1 kinase²¹ (a homologue of the mitotic cdc2 kinase²²). This peptide is an excellent substrate for starfish cdc2 kinase and competitively inhibits the phosphorylation of histone H1 by this enzyme. At a peptide concen-

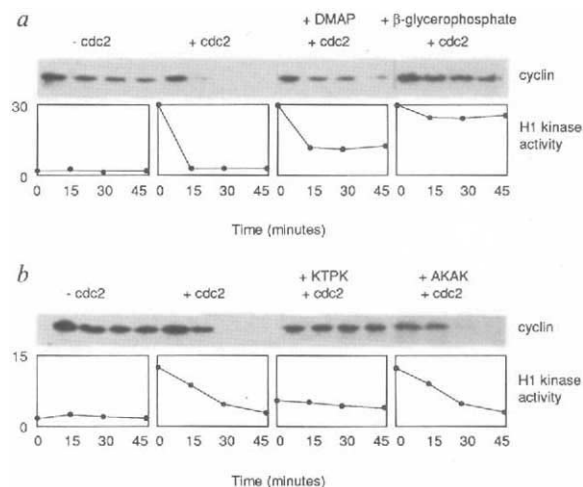


FIG. 2 Cyclin degradation is inhibited by 6-DMAP, β -glycerophosphate (β -gly) and KTPK peptide. The experiment was performed as described in Fig. 1. **a**, 1 mM DMAP or $20 \text{ mM magnesium-}\beta$ -glycerophosphate were added to the extract together with the cdc2 kinase ($30 \text{ U } \mu\text{l}^{-1}$ final concentration as measured in the extract at zero time); **b**, Synthetic H1 peptide KTPKKAKKPKTPKKAKKL²¹ (0.25 mM) or the control peptide AKAKKTPKAKK (0.6 mM) were added, together with $12 \text{ U } \mu\text{l}^{-1}$ cdc2 kinase. The histone H1 kinase activity (in units μl^{-1}) measured at time zero in the presence of KTPK peptide was reduced because this peptide partially inhibited the kinase activity of cdc2 even after dilution in the assay.

tration of 100 μM in assay buffer (that is, without egg extract), the phosphorylation of 1 mg ml^{-1} histone H1 by 5 U μl^{-1} of cdc2 kinase was inhibited by 80%. At 250 μM , we obtained 95% inhibition. In the concentrated egg extracts, 250 μM peptide inhibited the incorporation of ^{32}P into specific proteins catalysed by 15 U μl^{-1} of added cdc2 kinase, so that the pattern of protein phosphorylation in the extract looked like an interphase pattern¹⁵. The peptide concentration required to inhibit cdc2 kinase-dependent phosphorylation in the extracts seems high, as endogenous substrates for the kinase are probably present at much lower concentrations than 250 μM . But the extracts are very concentrated and the peptide may absorb to negatively charged components such as RNA and is probably degraded fairly rapidly. We found that 250 μM was the lowest concentration at which the peptide inhibited cdc2 kinase-induced cyclin degradation (Fig. 2b). As a control, we used a shorter peptide with a similar amino-acid composition: AKAKKTPKAKK. This peptide did not inhibit the H1 kinase activity of purified cdc2 kinase, did not inhibit cdc2 kinase-dependent phosphorylation in the crude extract and, even at 600 μM , did not prevent cdc2 kinase-induced cyclin degradation (Fig. 2b). We conclude that the protein kinase activity of p34^{cdc2} is essential for inducing cyclin degradation.

Certain compounds such as β -glycerophosphate salts stabilize MPF and histone H1 kinase activities from frog eggs^{17,23}. This might reflect the stabilization of cyclin against degradation. To test this idea we added 20 mM magnesium- β -glycerophosphate to the *in vitro* destruction assay. Figure 2a shows that this completely stabilized cyclin against degradation triggered by added cdc2 kinase. Moreover, the cdc2 kinase activity remained high in the presence of the added β -glycerophosphate. Although Erikson and Maller recently showed that β -glycerophosphate partially inhibited the kinase activity of purified p34^{cdc2} (ref. 24), this does not seem to be the case in crude extracts and we routinely assay histone kinase in 80 mM β -glycerophosphate; in fact, omitting it from the assay buffer tended to decrease the activity. Paradoxically, therefore, although the destruction of cyclin is initiated by a protein kinase, it could involve a protein phosphatase, as sodium- β -glycerophosphate is thought to act as a phosphatase inhibitor. Nevertheless, okadaic acid, a powerful inhibitor of phosphatases²⁵ type-1 and type-2A, did not inhibit the destruction of cyclin induced by cdc2 kinase in our system; if anything, it accelerated cyclin destruction²⁶. Thus if β -glycerophosphate inhibits cyclin degradation by inhibiting a phosphatase, the phosphatase cannot be of type-1 or type-2A, and it presumably acts downstream of cdc2 kinase in the pathway leading to cyclin proteolysis. Irrespective of the precise

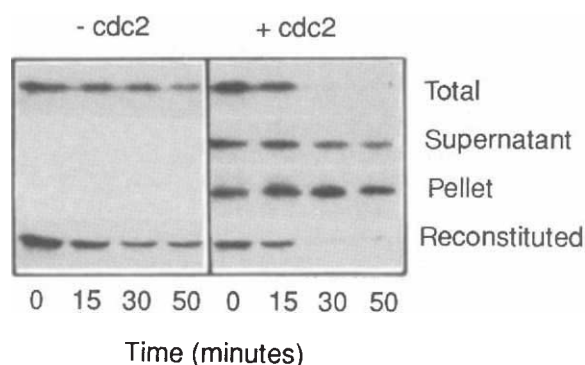


FIG. 3 cdc2 kinase-induced degradation of cyclin requires high M_r components. Cyclin was incubated in the complete reaction mix described in Fig. 1 (total), or with 150,000g supernatant depleted of high M_r material (supernatant), or with the pellet from this spin resuspended in acetate buffer (pellet), or a mixture of both (reconstituted), without (left panel) or with (right panel) 20 U μl^{-1} cdc2 kinase.

mechanism, these data suggest that β -glycerophosphate salts help stabilize MPF by preventing cyclin degradation.

We previously found that 'particulate material' that could be sedimented only in very high centrifugal fields was involved in the activation of cdc2 kinase¹⁵. As a first attempt to fractionate the cyclin destruction machinery we tested whether high-speed supernatants could support cyclin destruction. By themselves, neither the high-speed supernatant nor the pellet were capable of destroying cyclin when cdc2 kinase was added (Fig. 3). When the pellet and the supernatant were mixed to reconstitute the extract, however, destruction was restored. This suggests that at least two components besides cyclin and cdc2 are required for cyclin destruction, one of which is included in the particulate material.

A threshold concentration of cyclin is required to activate cdc2 kinase²⁷, probably by permitting or promoting post-translational modifications of p34^{cdc2}. The results presented here show that when cdc2 kinase activity exceeds a certain level, it triggers a series of reactions that lead to the proteolysis of cyclin. This is evidence for the existence of a negative feedback loop that could form the basis of a minimum cell-cycle oscillator, as shown in Fig. 4. The system cannot reach equilibrium; it keeps oscillating, because threshold levels of both cyclin protein and cdc2 kinase activities trigger post-translational reactions with built-in time delays, and the destruction of cyclin causes the loss of cdc2 kinase activity. The period of the oscillator is determined in part by the rate of cyclin synthesis and in part by the time constants of the slow reactions, with cyclin degradation as the ultimate irreversible step. Figure 4 describes a possible mechanism by which cdc2 kinase could trigger cyclin degradation. In this model, mitotic cdc2 kinase increases the phosphorylation of a protein, X, which in turn activates the protease that destroys cyclin. At the end of M phase, after cyclin has been destroyed, cdc2 kinase is inactivated and so is the proteolysis machinery. The termination of cyclin proteolysis is an important feature of the system, for otherwise the next cycle could never take place. An alternative model, not illustrated here, is suggested by the observation that cyclin itself is phosphorylated during mitosis, probably by cdc2 kinase^{5,7}. According to this model, alterations to cyclin

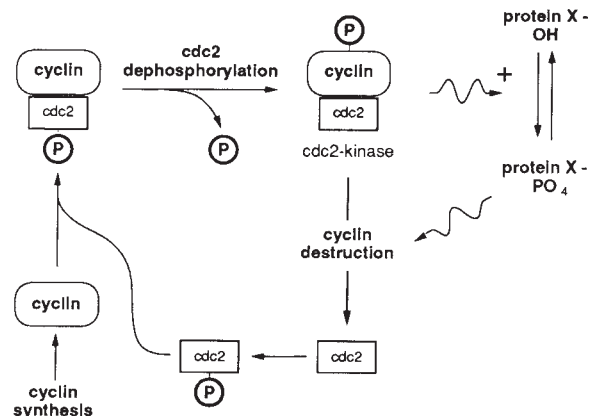


FIG. 4 A model to explain the oscillation of cdc2 kinase in early *Xenopus* embryos. The phosphorylated cdc2-cyclin complex shown at top left has no cdc2 kinase activity. It is activated by dephosphorylation of its p34^{cdc2} subunit. One of the cellular targets for cdc2 kinase is the hypothetical protein X. The phosphorylated form of protein X activates the protease that destroys cyclin. Once cyclin has been destroyed, cdc2 kinase is rapidly inactivated, either by rephosphorylation of the p34^{cdc2} subunit as shown here, or by combination with an inhibitor. The new synthesis of cyclin eventually permits reactivation of cdc2 kinase, presumably by promoting dephosphorylation of the p34^{cdc2} subunit. Cell-cycle oscillations in cdc2 kinase result from the continual accumulation of cyclin followed by its destruction, which is triggered by the active form of cdc2 kinase.

itself rather than to the protease would regulate the destruction of cyclin. Further work is necessary to distinguish between these possibilities. □

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New variation on the translocation of proteins during early biogenesis of apolipoprotein B

Steven L. Chuck^{*,†}, Zemin Yao[‡], Brian D. Blackhart[‡], Brian J. McCarthy[‡] & Vishwanath R. Lingappa^{*,§}

Departments of ^{*}Medicine, [†]Microbiology and Immunology, and [§]Physiology, and [‡]The Gladstone Foundation Laboratories for Cardiovascular Research, Cardiovascular Research Institute, University of California, San Francisco, California 94143, USA

APOLIPOPROTEIN B (apo B) is crucial for the transport of cholesterol in humans. It is a large secretory protein that mediates the uptake of low-density lipoproteins and renders several forms of lipid droplets soluble in the blood^{1–3}. The binding of lipid by apo B also prevents this hydrophobic protein from precipitating in aqueous solution⁴. In the endoplasmic reticulum, nascent secretory proteins must be translocated through an aqueous channel in the membrane into the aqueous lumen⁵, so some novel form of processing may be necessary to maintain the solubility of apo B during its translocation. We have discovered that the biogenesis of apo B in cell-free systems does indeed involve a new variation on protein translocation: unlike typical secretory proteins, apo B is synthesized as a series of transmembrane chains with large cytoplasmic domains and progressively longer amino-terminal regions that are protected against added proteases during the translocation process. In contrast to typical transmembrane proteins, these transmembrane chains are not integrated into the bilayer. Moreover, the transmembrane chains with the shortest protected domains are precursors of forms whose protection is progressively extended to cover the length of the protein. This stepwise conversion occurs post-translationally for the most part. We propose a model on the basis of these findings for the biogenesis of apo B.

Studying early events in the biogenesis of full-length apo B-100 (relative molecular mass of 550,000) is difficult *in vivo*

because of the lack of synchrony in synthesis and degradation of most of the chains⁶; also the size of apo B-100 makes its synthesis *in vitro* technically daunting. Lengths of apo B shorter than B-100, however, are also secreted by cells. In humans, intestinal cells secrete apo B-48, which constitutes the N-terminal 48% of B-100, on chylomicrons^{7,8}. In addition, a rare mutant species of apo B corresponding to the N-terminal 37% of B-100 (ref. 9) has been detected on lipoprotein particles in humans. Finally, lengths of apo B as short as the N-terminal 15% of apo B-100 are secreted from heterologous transfected hepatocytes (unpublished observation). Therefore, if any unusual translocational steps are involved in the synthesis of apo B, they are likely to occur during the synthesis of an amino-terminal domain of apo B. We therefore chose to study the N-terminal 15% of apo B-100 (termed apo B-15) in cell-free systems. Apo B-15 includes an N-terminal cleaved signal sequence of 27 amino acids and several hydrophobic regions that are representative of those occurring throughout apo B-100 (refs 10, 11) and which are assumed to bind to lipids. But these hydrophobic regions contain no more than 13 consecutive hydrophobic amino acids, and none constitutes a membrane-spanning domain¹⁰.

When synthesized in the presence of microsomal vesicles, apo B-15 is translocated across the membranes, as evidenced by glycosylation (Fig. 1a). After digestion with proteinase K, the majority of translocated chains are cleaved, yielding several discrete bands that are suggestive of a family of transmembrane proteins with different lengths protected against digestion (Fig. 1b, lane 4). Proteolysis with trypsin and proteinase K together did not change this pattern of bands (data not shown). Immunoprecipitation of the protease-digested translation products with antipeptide sera directed against amino-acid residues 12–27 of mature apo B showed that these bands represent different N-terminal lengths of apo B (Fig. 1c). These N-terminal lengths are protected by virtue of the translocation process, rather than any intrinsic resistance to protease or nonspecific interaction with the membrane, because apo B-15 is completely digested (1) in the absence of vesicles; (2) in the presence of vesicles solubilized with detergent; (3) in the presence of membranes rendered incompetent for translocation by pretreatment with *N*-ethyl maleimide (Fig. 1b), or (4) in the presence of membranes depleted of the signal recognition particle (data not shown). These results strongly suggest that the amino-terminal region of apo B is synthesized as a transmembrane protein, in contrast to conventional secretory proteins.

Unlike typical integral membrane proteins, however, these transmembrane forms of apo B are not integrated into the bilayer. An aliquot of apo B-15 synthesized in the presence of microsomal vesicles (under conditions identical to those used for Fig. 1b) was mixed with aliquots of the simple secretory protein, bovine prolactin, and an integral transmembrane protein called S-gG-ST-P (a chimaeric protein that includes the IgM stop-transfer sequence¹²), each of which was translated *in vitro* independently in the presence of microsomal membranes. The pooled sample was divided in half and treated with either Tris-buffered sucrose (pH 7.5) or 0.1 M sodium carbonate (pH 11.5) before centrifugation to distinguish integral from peripheral proteins¹³. As expected, all three proteins were found in the pelleted membranes when treated with Tris-buffered sucrose, the simple secretory protein was extracted by carbonate, whereas the integral transmembrane protein was retained in the bilayer. Apo B-15, however, was extracted from the membrane in amounts comparable to that of the simple secretory protein (Fig. 2).

Finally, we used pulse-chase analysis to show that the various transmembrane forms were progressive intermediates in the formation of fully protected apo B-15. First, we found that 16–18 min are necessary for a chain of apo B-15 to be completely synthesized in the wheat-germ translation system supplemented with membranes (data not shown). Translation of apo B-15 was

then initiated in the presence of membranes and [35 S]cysteine, and after 10 min labelling, a 1,000-fold excess of unlabelled cysteine was added. As shown in Fig. 3, the transmembrane form with the shortest protected domain predominates early in the incubation, then progressively decreases. Conversely, the fully protected chains accumulate steadily, reaching a maximum late in the incubation. The forms having intermediate lengths protected are maximal mid-way through the incubation. We conclude that this progressive conversion to the fully protected forms occurs mainly post-translationally, because synthesis of the last chains labelled in our 10-min pulse should be complete by 30 min.

Our results suggest a model for the biogenesis of apo B-15 (Fig. 4). First, nascent apo B-15 is targeted to the membrane of the endoplasmic reticulum (ER)^{14,15} and a short length of the nascent chain (a) is translocated into the lumen (step 1). Second, translocation of the domain immediately downstream of the first transmembrane region (domain b, Fig. 4) stops while elongation proceeds. The growing chain might cross the membrane at one or several discrete points (c, e in step 2). Third, translation is completed (a-f, step 3). In the figure, the ribosome is released,

but subsequent events may depend on its attachment. At this point, added proteases will digest the cytoplasmic domains, leaving only the shortest translocated domain (and other tiny luminal fragments if the chain crosses the bilayer at more than one point, as in region c in step 3). After translation, successive domains become protected from proteases: at this stage, protease digestion reveals that several N-terminal fragments are protected which become progressively longer (step 4). This stepwise conversion may be due to progressive translocation of C-terminal domains into the lumen (as shown in step 4), or it may result from conformational changes in the transmembrane chains that render protease-susceptible sites inaccessible. Lastly, the chain becomes fully protected, either as a result of complete translocation into the lumen (step 5, top) (the chain may remain associated with the inner leaflet of the bilayer), or because it acquires a transmembrane conformation that makes the entire chain resistant to proteases (step 5, bottom).

Previously, nascent apo B was assumed to be translocated completely across the ER membrane co-translationally, whereupon it became associated with the inner leaflet rather than spanning the bilayer^{1,16}. This hypothesis stemmed from

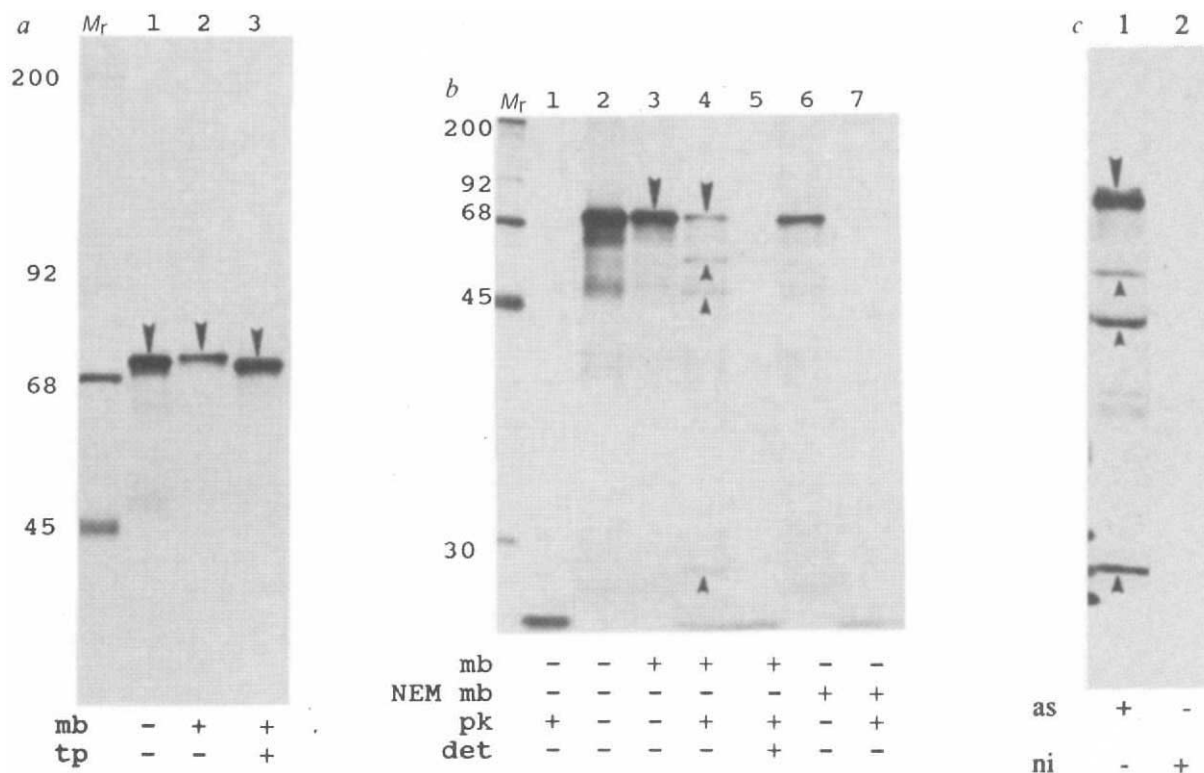


FIG. 1 *a*, Expression of Apo B-15 *in vitro*. Apo B-15 was translated in the absence (lane 1), or presence (lanes 2 and 3) of microsomal membranes (mb). In lane 3, a tripeptide (Asn-Tyr-Thr) competitor for *N*-linked glycosylation (tp) was added at the start of translation. Downward-pointing arrows indicate the principal products of each reaction displayed by SDS-PAGE on 5–12% gradient gel. Molecular weight markers (M_r) from 45,000–200,000 (45–200K) are shown in the left lane. *b*, Proteolysis studies of Apo B-15. Apo B-15 was translated for 90 min in the absence (lanes 1 and 2) or presence of microsomal membranes (mb; lanes 3–5), or in the presence of vesicles pretreated with *N*-ethyl maleimide (NEM mb; lanes 6 and 7). After translation, the samples were incubated on ice for 30 min either alone (lanes 2, 3 and 6), with 0.4 mg ml⁻¹ proteinase K (pk; lanes 1, 3 and 7), or with proteinase K and 0.5% Triton X-100 (det; lane 5) before boiling in SDS. Aliquots were then displayed on a 12–17% gradient gel by SDS-PAGE and autoradiography. In lane 4, the downward-pointing arrow indicates the fraction of chains that are fully protected from digestion by proteinase K (compare with the band indicated by the arrow in lane 3). The upward-pointing arrows indicate the principal fragments that are partially protected from

digestion by proteinase K. Molecular weight markers (M_r) from 30–200K are shown in the left lane. *c*, Immunoprecipitation of the fragments of apo B-15 partially protected from digestion by proteinase K. Lane 1, Immunoprecipitation with anti-peptide sera (as) to amino acids 12–27 of apo B-100. The upward-pointing arrows indicate the principal transmembrane fragments protected from digestion by proteinase K. The downward-pointing arrow indicates full length apo B-15. Lane 2, Non-immune serum (ni). **METHODS.** The complementary DNA encoding the N-terminal 15% of apo B-100 (ref. 10) followed by a termination codon (TAG), was engineered downstream of the SP6 promoter and expressed by transcription-linked²⁴ translation in a wheat germ cell-free system for 90 min in the presence or absence of vesicles derived from the ER of canine pancreas^{23,24}. Aliquots of the [35 S]methionine-labelled products of translation were digested with proteinase K with or without nonionic detergents to solubilize the vesicles²⁵. After boiling in sodium dodecyl sulphate (SDS), the samples were analysed by polyacrylamide gel electrophoresis in SDS (SDS-PAGE) followed by autoradiography.

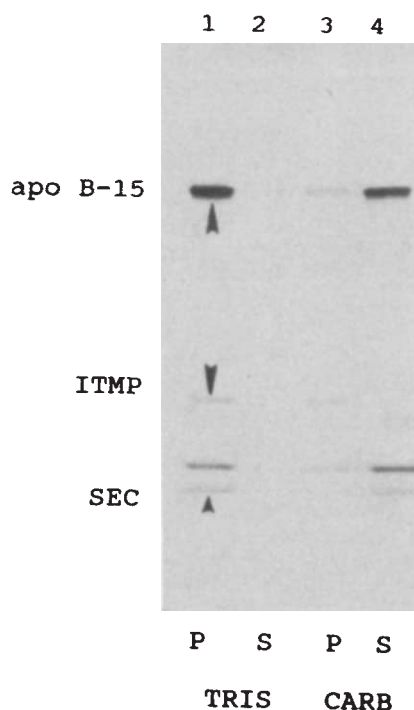


FIG. 2 Carbonate extraction of apo B-15. The simple secretory protein bovine prolactin (SEC), the glycosylated integral transmembrane protein (ITMP) S-gg-ST-P (see text), and apo B-15 were synthesized independently *in vitro* in the presence of microsomal membranes and then mixed. The pooled sample was divided in half and diluted 100-fold with either Tris-buffered 0.25 M sucrose, pH 7.5 (TRIS), or 0.1 M sodium carbonate, pH 11.5 (CARB) and incubated on ice for 30 min. After centrifugation at 50,000 r.p.m. in a Beckman-Ti-70.1 rotor at 4°C for 2 h, proteins in the supernates (S) were precipitated with 15% trichloroacetic acid, washed with ethanol/ether, then dissolved in SDS; pellets (P) were also dissolved in SDS. Aliquots were then displayed by SDS-PAGE on a 5–12% gradient gel followed by autoradiography. The processed form of prolactin (SEC) is indicated by the small upward-pointing arrow, the glycosylated form of S-gg-ST-P (ITMP) by the downward-pointing arrow, and apo B-15 by the large upward-pointing arrow.

FIG. 3 Pulse-chase analysis of apo B-15. Apo B-15 was translated *in vitro* in the presence of microsomal vesicles and [35 S]cysteine. At 10 min, a 1,000-fold excess of unlabelled cysteine was added. (The effectiveness of the 1,000-fold dilution was demonstrated in a separate experiment in which either unlabelled cysteine or water was added to two similar ongoing translations of apo B-15, followed 10 min later by the addition of prolactin transcript. Densitometry revealed that the synthesis of radiolabelled prolactin was reduced by > 95% after dilution with unlabelled cysteine compared with water; data not shown.) Samples were removed at 20, 30, 45, 60 and 90 min for analysis by proteolysis followed by SDS-PAGE and autoradiography. The proportion of chains in each transmembrane band was quantitated by densitometry corrected for the distribution of cysteines. The values for the smallest band (squares, shortest), all other transmembrane bands (triangles, intermediate length), and the band corresponding to fully protected chains (filled circles) are plotted.

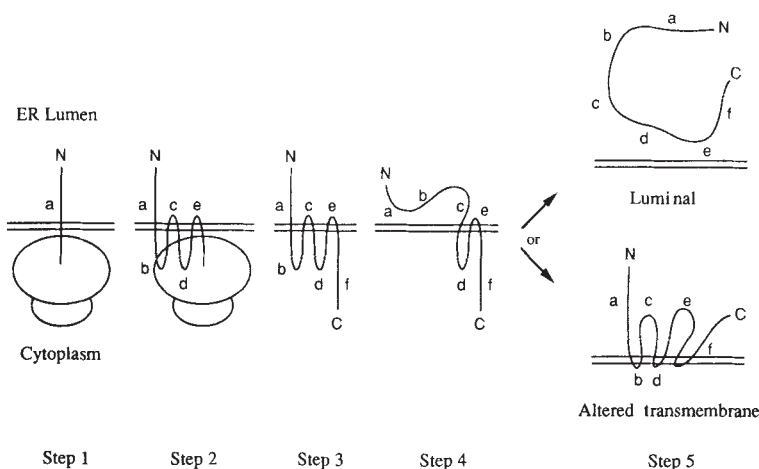
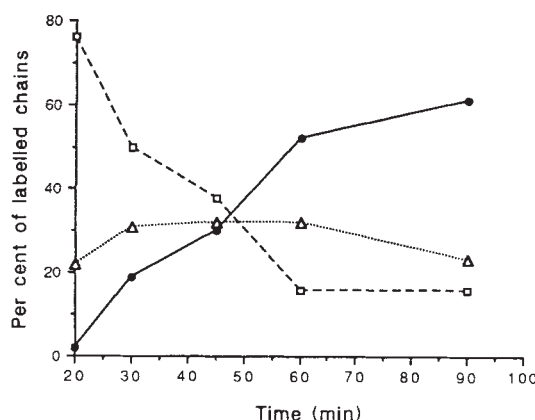


FIG. 4 Model of the biogenesis of apo B-15 (see text for details). N and C denote the amino and carboxy termini of the protein.

observations that apo B contains no more than 13 consecutive hydrophobic amino acids^{10,11}—fewer than the minimum considered necessary to span a bilayer—yet nascent apo B is associated with the membrane of the ER^{17,18}. But our results indicate that during its biogenesis, apo B-15 is a transmembrane protein with unusual features, suggesting that the N-terminal 15% of nascent apo B-100 might also span the bilayer without being integrated into it.

The assembly of apo B containing lipoproteins may begin within the membrane of the ER^{16,17}. It is tempting to speculate that the stepwise, post-translational lengthening of the protease-resistant N-terminal domain of apo B described here is necessary for the progressive assembly of the protein with lipid from the ER membrane. Thus the translocation of some proteins may be coupled to their assembly: the membrane of the ER may participate in the assembly of apo B and other complex proteins or particles, rather than acting simply as a barrier between intracellular compartments. Alternatively, the delay in apo B translocation may reflect the unfavourable kinetics of translocating such a hydrophobic secretory protein.

These unusual translocational steps may be involved in the regulation of the biogenesis of apo B. Control of apo B secretion occurs after transcription¹⁹ and probably after translation, because most of the apo B synthesized seems to be degraded in cultured hepatocytes⁶. Chains of apo B that do not complete the multistep process of translocation described here may remain in the membrane, destined for a degradative^{20,21} rather than a secretory pathway.

Transmembrane proteins are usually integrated in the membrane by the action of stop-transfer sequences, but proteolysis and carbonate extraction of apo B-15 indicates that stop-transfer and integration might be dissociated. Perhaps discrete topogenic sequences will be identified that mediate each of these normally coupled events in the biogenesis of transmembrane proteins. Thus, apo B could contain sequences that direct stop-transfer but which have insufficient consecutive hydrophobic amino acids to mediate integration¹⁴.

The relationship between the transmembrane chains and the translocational machinery²² is unknown. These intermediates span the membrane without being integrated into it, and they ultimately appear fully protected and probably completely translocated. Thus, the transmembrane intermediates may still be associated with translocational machinery, in a state in which translocation is arrested but may subsequently proceed. □

Widespread distribution and fitness contribution of *Xanthomonas campestris* avirulence gene *avrBs2*

Brian Kearney & Brian J. Staskawicz

Department of Plant Pathology, University of California, Berkeley, California 94720, USA

DISEASE-resistance genes introduced into cultivated plants are often rendered ineffective by the ability of pathogen populations to overcome host resistance^{1–3}. The bacterial pathogen *Xanthomonas campestris* pathovar *vesicatoria* causes bacterial spot disease of tomato and pepper, and this pathogen has been shown to overcome disease resistance in pepper (*Capsicum annuum*) by evading the recognition and defence response of the host plant^{4–6}. Numerous resistance genes to bacterial spot have been identified in pepper and its wild relatives, each providing resistance to specific races of *X. c. vesicatoria*^{7–13}. The resistance gene *Bs1*, for example, provides resistance to *X. c. vesicatoria* strains expressing the avirulence gene *avrBs1* (ref. 11); *Bs2* provides resistance to strains expressing *avrBs2* (ref. 13) and so on. We now report that *avrBs2* is highly conserved among strains of *X. c. vesicatoria*, and among many other pathovars of *X. campestris*. Furthermore, we find that *avrBs2* is in fact needed for full virulence of the pathogen on susceptible hosts. This implies that plants carrying *Bs2* can recognize an essential gene of the bacterial pathogen, which may explain why *Bs2* confers the only effective field resistance to *X. c. vesicatoria* in pepper.

In contrast to other *X. c. vesicatoria* avirulence genes which have been cloned¹³, the widespread distribution of *avrBs2* is striking. Each of the other identified *X. c. vesicatoria* avirulence genes has a restricted distribution among the physiological races of the bacterial species^{11–13}, but *avrBs2* is found in all strains of the tomato and pepper races of *X. c. vesicatoria* tested, including the 20 representative strains we commonly use (data not shown) and over 500 other strains (R. E. Stall, personal communication).

The distribution of *avrBs2* extends to other pathovars of *X. campestris* that do not cause disease on pepper. When inoculated on pepper, *X. campestris* pathovars other than *X. c. vesicatoria* generally induce a hypersensitive defence response¹⁴ on both pepper cultivars ECW (lacking *Bs2*) and ECW20R (homozygous for *Bs2*). As each resistance gene–avirulence gene interaction in pepper causes a phenotypically unique hypersensitive response^{13,15}, the *avrBs2* activity of each pathovar could be tested. The typical *avrBs2*–*Bs2* hypersensitive response is a light brown confluent necrosis that appears 12–24 h after inoculation

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TABLE 1 Distribution of *avrBs2* activity and DNA homology within *Xanthomonas*

Strain	Hypersensitivity on ECW20R	<i>avrBs2</i> Homology
<i>X. c. vesicatoria</i>	yes	yes
<i>X. c. alfalfae</i>	yes	yes
<i>X. c. phaseoli</i>	yes	yes
<i>X. c. malvacearum</i>	yes	yes
<i>X. c. campestris</i>	yes	yes
<i>X. c. vitians</i>	yes	yes
<i>X. c. vignicola</i>	yes	yes
<i>X. c. glycines</i>	no	yes
<i>X. c. holcicola</i>	no	yes
<i>X. c. oryzae</i>	—	yes
<i>X. c. citri</i>	—	yes
<i>X. fragariae</i>	no	no

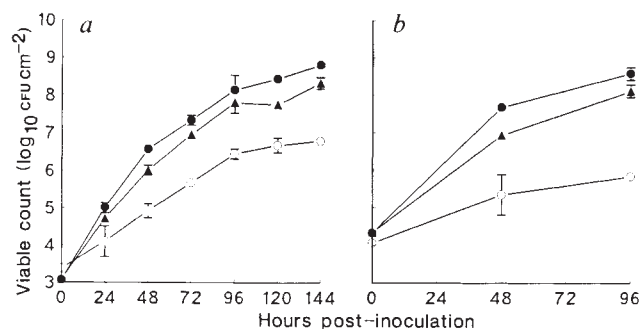


FIG. 1 a, Growth of *X. c. vesicatoria* strain 82-8 (●) and the strain with a spontaneous mutation at *avrBs2* (○) in the susceptible pepper cultivar ECW. The mutant strain displayed reduced virulence in the plant when compared to the wildtype; 5×10^6 CFU cm⁻² and 5×10^8 CFU cm⁻², respectively. Complementation of the mutant with a plasmid-borne copy of *avrBs2* allowed the growth of the mutant to approach the wild-type level (▲). b, Growth of *X. c. alfalfae* (●) and a mutant carrying a marker-exchange mutation in *avrBs2* (○) on the susceptible alfalfa cultivar Moapa 69. The mutation at *avrBs2* reduces the ability of *X. c. alfalfae* to multiply in the susceptible host. Growth of the *X. c. alfalfae* exchange mutant carrying *avrBs2* from *X. c. vesicatoria* (▲) nears the wild-type growth level, indicating that the *X. c. vesicatoria* *avrBs2* clone complements the virulence of the alfalfa pathogen back to its wild-type level.

METHODS. Mean log₁₀ CFU cm⁻² for both growth curves was determined essentially as described in ref. 6. The standard error for data points without visible error bars is less than 0.10 log₁₀ CFU cm⁻².

on ECW20R plants and is unlike any of the hypersensitive responses controlled by other pepper genes¹³. The pathogens that induced *Bs2* hypersensitivity specifically on ECW20R were classified as having *avrBs2* activity. Strains of *X. c. pathovars alfalfae*, *malvacearum*, *vignicola*, *vitians*, *campestris* and *phaseoli* display *avrBs2* activity (Table 1).

Evidence of *avrBs2* conservation was confirmed by Southern analysis¹⁶. For each pathovar, DNA probed with the cloned *avrBs2* gene from *X. c. vesicatoria* under stringent conditions (65 °C, 0.1 × SSC buffer, 0.1% SDS) revealed a conserved 2.35 kilobase (kb) *SphI* fragment (Table 1). The pathovars *X. c. holcicola* and *X. c. glycines* have no *avrBs2* activity but display DNA homology to *avrBs2* nevertheless. In these cases, it is not known whether the *avrBs2*-homologous sequences are inactive or whether *avrBs2* inhibitors are acting in *trans*.

Although inoculation and DNA hybridization studies strongly suggest *avrBs2* activity in the other pathovars, proof of conservation was provided by cloning the *avrBs2*-homologous sequences of two of the pathovars. Libraries of *X. c. alfalfae* and *X. c. phaseoli* were made in the vector pUC119 (ref. 17). Colony hybridization with the *X. c. vesicatoria* *avrBs2* gene identified homologous clones in each library. The cloned inserts were transferred into the wide host-range vector pLAFR3 (ref. 18) and conjugated into a *X. c. vesicatoria* strain containing a mutation at *avrBs2* that renders the strain virulent on ECW20R plants. In both cases, the cloned gene complemented the *avrBs2* mutation and restored avirulence on ECW20R plants. The ability of the *X. c. alfalfae* and *X. c. phaseoli* clones to complement the *X. c. vesicatoria* *avrBs2* mutation confirmed that gene activity as well as DNA homology is conserved among these pathovars.

Conservation of *avrBs2* among all *X. c. vesicatoria* strains implies that *avrBs2* plays an important part in the fitness of the pathogen, a hypothesis tested by comparing the growth of *X. c. vesicatoria* with either a wild-type or mutant *avrBs2* gene in the susceptible host ECW. The wild-type strain grew to $\sim 5 \times 10^8$ colony-forming units (CFU) cm⁻² in the plant after 4 days. In the same period of time, the mutant grew to 5×10^6 (Fig. 1a), roughly the same level of growth as that of the wild-type bacterium in the resistant host (data not shown). When the mutation was complemented by a plasmid-borne copy of *avrBs2*, the

virulence of the mutant was restored to near wild-type levels (Fig. 1a), showing that full virulence depends on *avrBs2*. In repeated testing, the spontaneous mutant was not complemented to full wild-type growth levels (Fig. 1a), suggesting that additional mutations affecting virulence may be present in the strain.

Conservation of *avrBs2* among the other *X. campestris* pathovars implies a role for *avrBs2* in the fitness of these pathogens as well. To test the hypothesis, a marker-exchange mutation was made in the *avrBs2*-homologous sequence from *X. c. alfalfae*, and growth of the wild-type and mutant strains in a susceptible alfalfa cultivar determined (Fig. 1b). The results indicate that mutation of the *avrBs2*-homologous sequence reduces the virulence of the alfalfa pathogen on its susceptible host by over 100-fold. Complementation of the *X. c. alfalfae* mutation with the *X. c. vesicatoria* avirulence gene restored the alfalfa pathogen to its former growth level, confirming the importance of *avrBs2* to the fitness of *X. c. alfalfae*.

Just as mammalian immunity is most effective when directed against highly conserved epitopes important to the fitness of a pathogen, we believe *Bs2* resistance has yet to be overcome in the field because *Bs2*-containing plants recognize a highly conserved bacterial signal involved in virulence. Mutations undoubtedly occur at *avrBs2*, but the reduced ability of the mutants to colonize *Bs2* plants prevents disease development. It is possible that *Bs2* resistance may break down in the field due to secondary mutations that restore virulence to the *avrBs2* mutants. Although in limited laboratory testing these types of mutations have not been recovered (data not shown), long-term field testing is required to confirm the durability hypothesis. The results presented here suggest that it may be possible to predict the durability of resistance genes in the future by analysing the conservation and importance of the bacterial signals they recognize.

Conservation of *avrBs2* among other *X. campestris* pathovars also suggests solutions to severe disease problems in other plant species; there is no effective genetic resistance in commercial tomatoes to *X. c. vesicatoria*, in bean to *X. c. phaseoli*, in cabbage to *X. c. campestris*, or in citrus to *X. c. citri*. When the *Bs2* gene is cloned and transferred to these other plant species, it may provide stable genetic resistance to these other diseases as well. □

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Highly conserved core domain and unique N terminus with presumptive regulatory motifs in a human TATA factor (TFIID)

Alexander Hoffmann, Eric Sinn, Tohru Yamamoto, Josephine Wang, Ananda Roy, Masami Horikoshi & Robert G. Roeder

Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, New York 10021, USA

THE factor TFIID is one of several general factors that are necessary and sufficient for transcription initiation by mammalian RNA polymerase II^{1,2}. Stable interactions with the common TATA element^{3,4} lead both to template commitment and to the assembly of the other general factors into a functional preinitiation com-

plex^{5,6}. Consistent with its key role in the promoter activation pathway, human TFIID also seems to be a target for some regulatory factors, as evidenced both by physical^{7,8} and functional⁹⁻¹² studies of interactions between these components. The evolutionary conservation of functional properties^{13,14} led to the purification and cloning of yeast TFIID¹⁵⁻¹⁹, the identification of presumptive structural motifs^{15,19}, and direct structure-function studies²⁰. Here we report the cloning of a complementary DNA encoding a functional human TFIID. This reveals an evolutionarily conserved core which corresponds precisely to the 180-residue DNA binding/activation domain determined²⁰ for yeast TFIID, a near absolute conservation of component structural motifs (direct repeats, central basic core/lysine repeat, and sigma homology), providing further support for their functional importance, and a unique N-terminal structure that suggests involvement in species-specific regulatory factor interactions.

Overlapping human TFIID cDNAs (Fig. 1) were cloned following polymerase chain reaction (PCR) amplification with minimally degenerate primers that were chosen from the yeast TFIID core domain previously shown to be necessary and sufficient for DNA binding²⁰ and to be conserved in *Schizosac-*

FIG. 1 Nucleotide and predicted amino-acid sequence of human TFIID cDNA. Shown is the combined sequence of three independent overlapping cDNA clones. The PCR product (nucleotide positions 651-862) contained in each of these is underlined with a solid line and sequences encoded by the primers with dashed arrows. The 1,005-base pair (bp) open reading frame is defined by translation start and stop codons (boxed in the nucleotide sequence) and encodes a polypeptide containing the characteristic TFIID homology core domain (amino acids 155-335, boxed) as well as an N-terminal region unique to this human TFIID polypeptide. A putative polyadenylation signal near the 3' end is underlined.

METHODS. A novel computer program was used to calculate the minimum degeneracy of oligonucleotides necessary to specify five amino-acid stretches within the previously mapped TFIID core domain²¹. These were used as primers to amplify DNA fragments by PCR from a Namalwa cell cDNA library²⁸. Five candidate products were selected on the basis of expected size and cross-hybridization to yeast TFIID probes at standard low stringency conditions²¹. Sequencing of the subcloned fragments revealed one that encoded a polypeptide with a high sequence homology to amino acids 91-161 of the *S. cerevisiae* TFIID. This insert was used to screen 10⁶ recombinants from the same library under standard high stringency conditions²¹. Three independent clones of 1,258, 1,254 and 1,232 bp were sequenced in both directions using the chain termination method. One contained a poly(A) tail 685 bp 3' of the TFIID ORF stop codon, whereas another contained the full ORF as evidenced by a start codon (ATG) in the correct reading frame preceded by stop codons (boxed) in the same reading frame.

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1      CTGAGAAGGGTGTGCTGGAGATGCTCTAGGAAAAATTGAATAGTGGACGAGTTCACGCCAAGGGTTTCTGGT
76     TTGCCAAGAAGAAAGTGAACATC ATG GAT CAG AAC AAC AGC CTG CCA CCT TAC GCT CAG GGC TTG
1      M D Q N N S L P P Y A Q G L
141    GCC TCC CCT CAG GGT GCC ATG ACT CCC GGA ATC CCT ATC TTT AGT CCA ATG ATG CCT TAT
15     A S P Q G A M T P G I P I F S P M M P Y
201    GGC ACT GGA CTG ACC CCA CAG CCT ATT CAG AAC ACC AAT AGT CTG TCT ATT TTG GAA GAG
35     G T G L T P Q P I Q N T N S L S I L E E
261    CAA CAA AGG CAG CAG CAG CAA CAA CAA CAG CAG CAG CAG CAG CAG CAG CAG CAG CAA CAG
55     Q Q R Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q
321    CAA CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAA CAG
75     Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q
381    GCT GCA GCC GTT CAG CAG CAG TCA ACG TCC CAG CAG GCA ACA CAG GGA ACC TCA GGC CAG GCA
95     A A A V Q Q S T S Q A T Q G T S G G C A A
441    CCA CAG CTC TTC CAC TCA CAG ACT CTC ACA ACT GCA CCC TTG CCG GGC ACC ACT CCA CTG
115    P Q L F H S Q T L T T A P L P G T T P L
501    TAT CCC TCC CCC ATG ACT CCC ATG ACC CCC ATC ACT CCT GCC ACG CCA GCT TCG GAG AGT
135    Y P S P M T P M T P I T P A T P A S E S
561    TCT GGG ATT GTA CCG CAG CTG CAA AAT ATT GTA TCC ACA GTG AAT CTT GGT TGT AAA CTT
155    S G I V P P Q L Q N I V S T V N L G C K L
621    GAG CTA AAG ACC ATT GCA CTT CGT GCC CCA AAC GCC GAA TAT AAT CCC AAG CGG TTT CCT
175    D L K T I A L R A R N A E Y N P K R F A
681    GCG GTA ATC ATG AGG ATA AGA GAG CCA CGA ACC ACG GCA CTG ATT TTC AGT TCT GGG AAA
195    A V I M R I R E P R T T A L I F S S G K
741    ATG GTG TGC ACA GGA GCC AAG AGT GAA GAA CAG TCC AGA CTG GCA GCA AGA AAA TAT CCT
215    M V C T G A K S E E Q S R L A R K Y A
801    AGA GTT GTA CAG AAG TTG GGT TTT CCA GCT AAG TTC TTG GAC TTC AAG ATT CAG AAC ATG
235    R V V Q K L G F P A K F L D F K I Q N M
861    GTG GGG AGC TGT GAT GTG AAG TTT CCT ATA AGG TTA GAA GGC CTT GTG CTC ACC CAC CAA
255    V G S C D V K F P I R L E G L V L T H Q
921    CAA TTT AGT AGT TAT GAG CCA GAG TTA TTT CCT GGT TTA ATC TAC AGA ATG ATC AAA CCC
275    Q F S S Y E P E L F P G L I Y R M I K P
981    AGA ATT GTT CTC CTT ATT TTT GTT TCT GGA AAA GTT GTA TTA ACA GGT GCT AAA GTC AGA
295    R I V L L I F V S G K V L T T G G A K V R
1041   GCA GAA ATT TAT GAA GCA TTT GAA AAC ATC TAC CCT ATT CTA AAG GGA TTC AGG AAG AGG
315    A E I Y E A F E N I Y P I L K G F R K T
1101   ACG TAATGGCTCTCATGTACCTTGCCTCCCCACCCCTCTTTTTTTTTTTTAAACAAATCAGTTTGTGTTGTA
355    TOCR
1179   CCTTTAAATGGTGGTGTGTTGAGAAGATGGATGTTGAGTTGCAGGGTGTGGCACCAGGTGATGCCCTTCTGTAAGTGGC
1258   CACCGCGGGATGCCGGGAAGGGGCATTATTTGTGCACTGAGAACACCGCGCAGTGACCTGTGAGTTGCTCATACCGTG
1337   CTGCTATCTGGGCAGCGCTGCCCATTTATTTATATGTAGATTTTAAACACTGCTGTGACAAGTTGGTTTGAGGGAACA
1416   AACTTTAAGTGTTAAAGCCACCTCTATAATTGATTGGACTTTTAAATTTAATGTTTTCCCATGAACACAGTTT
1495   ATATTTCTACAGAAAAGTAAATCTTTTTTAAAGTGTGTTTTCTAATTTATACTCCTAGGGTTATTTCTGTG
1574   CCAGACACATTCACCTCTCCAGTATTGCAGACAGAATATATGTGTTAATGAAATGAATGGCTGTACATATTTTTTT
1653   CTTTCTTCAGAGTACTCTGTACAAATAATGCAGTTTATAAAAAAAAAAAAAAAAAAAAA 1710

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Saccharomyces pombe TFIID²¹. The combined sequence contains a 335-residue open reading frame encoding a polypeptide of calculated relative molecular mass (M_r) 37,160. Southern blots with short RNA probes showed hybridization with several high M_r placental DNA fragments, indicative either of several homologous genes or of a complex intron-exon structure (as observed for *Schiz. pombe*²¹ and *Arabidopsis*²²), whereas RNase protection assays indicated a low abundance of uniform length transcripts (data not shown).

Several assays showed that the cloned cDNA encodes a functional TFIID with TATA-box-binding and basal-level-transcription activities. A polypeptide (p38) of apparent M_r 38,000 was generated from expression of the cDNA in bacteria (data not shown) or translation of cDNA-derived transcripts in a reticulocyte lysate (Fig. 2a). Polypeptide p38 bound specifically to the TATA element of the adenovirus ML promoter as shown by the different sensitivities of two p38-dependent complexes to oligonucleotides with normal or mutant TATA sequences (Fig. 2b). One of these complexes was indistinguishable from the complex formed with natural human TFIID, whereas the second had a faster mobility; this suggests that the translated p38 protein may not be structurally equivalent to natural TFIID and/or that p38 may interact with or be modified by factors in the lysate.

When analysed in a cell-free transcription system reconstituted with general factors from HeLa cells, both the lysate-derived (Fig. 2c) and the bacterially expressed (Fig. 2d) human p38, as well as bacterially expressed yeast TFIID (Fig. 2d), could mediate basal level transcription as effectively as natural human TFIID. In contrast, although the stimulatory effect of the human transcriptional activator USF (which binds upstream

of TATA on the ML promoter³) was readily apparent with native human TFIID, no detectable enhancement was observed with the bacterially expressed yeast or human TFIID polypeptides (Fig. 2d). Possibly related to this difference, the native human TFIID showed transcripts in the guanosine(G)-less cassette assay indicative of multiple initiations on the same template (Fig. 2d legend), whereas the bacterially expressed proteins did not.

Figure 3b details, and Fig. 4 summarizes schematically, a comparison of the human TFIID sequence with TFIID sequences from *Saccharomyces cerevisiae*, *Schiz. pombe*²¹, *Arabidopsis thaliana*²² and *Drosophila melanogaster* (M. Muhich, C. Iida and C. Parker, unpublished observations). There is a remarkable degree of sequence conservation ($\geq 80\%$ identity) and complete colinearity between the ~ 180 -residue C-terminal regions of the factors. Moreover, this region correlates exactly with the region of *S. cerevisiae* TFIID previously shown²⁰ to be necessary and sufficient for TATA box binding and core promoter transcription. Altogether, these observations clearly argue for a conserved TFIID 'core' which explains the evolutionary conservation of TFIID function.

The TFIID core also contains the structural motifs (Fig. 3b, Fig. 4) noted previously^{15,19} and in the accompanying paper²¹. The residues comprising these elements show an even more striking (near absolute) conservation from yeast through to humans, which argues strongly for a conservation of functional roles as follows. First, there are direct repeats¹⁹ which allow an element of symmetry in the folded TFIID molecule. This has been proposed to be important for DNA binding, both by analogy to the dimeric structure of other site-specific DNA binding proteins and on the basis of mutational analysis²⁰ (T.Y.,

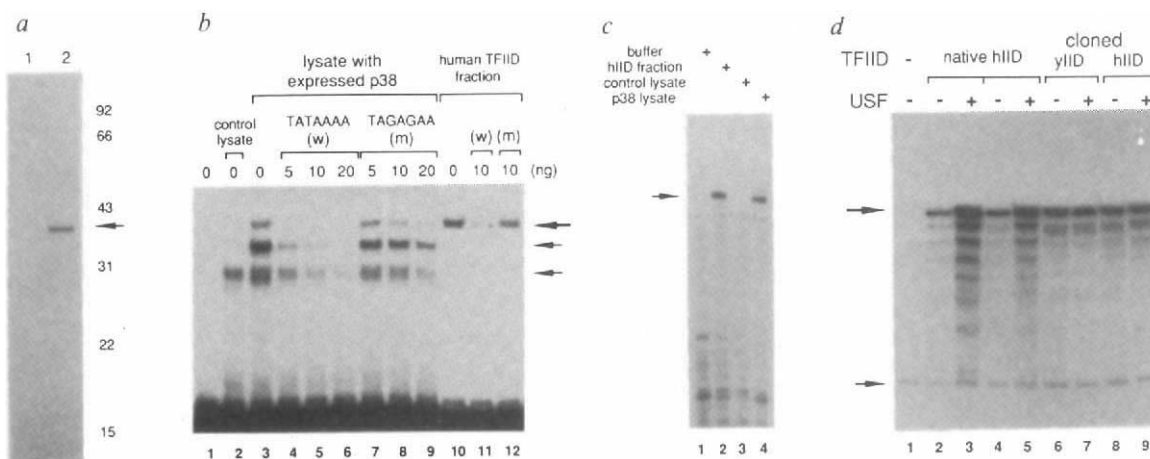


FIG. 2 *In vitro* expression and function of the human cDNA-encoded TFIID. **a**, SDS-PAGE analysis of [³⁵S]methionine-labelled TFIID produced in reticulocyte lysate. Lysates were programmed with no RNA (lane 1) or human TFIID cDNA-derived mRNA (lane 2). The size of the labelled TFIID polypeptide (arrow) based on size markers (not shown) is M_r 38,000. **b**, Site-specific binding of expressed and native human TFIID. Gel shift assays with a major late promoter (MLP) fragment contained: lane 1, no protein; lane 2, control lysate with no RNA; lanes 3-9, lysate programmed with human TFIID mRNA; lanes 10-12, amino-octyl fraction of native human TFIID⁴. Unlabelled oligonucleotides (MLP, positions -45 to -15) contained either a wild type (w) or a mutant (m) TATA box (as indicated) and were added as competitors in the nanogram amounts indicated above each lane. The bold arrow and the upper thin arrow indicate human TFIID specific complexes whereas the lower thin arrow indicates a nonspecific complex formed by a component in the lysate. **c**, Transcriptional activity of expressed human TFIID in a reconstituted system. Reactions containing an MLP template (pSmaF) and partially purified factors TFIIB, TFIIE/F and RNA polymerase II²⁹ were complemented with buffer (lane 1), DE52 fraction of human TFIID⁴ (hIID) (lane 2), unprogrammed lysate (lane 3), and lysate programmed with human TFIID mRNA (lane 4). Specific transcripts measured by primer extension are indicated by the arrow. **d**, USF-stimulated transcription with human TFIID. Reactions containing

an adenovirus MLP template with upstream sequences (-400 to +10) attached to the 380-bp G-less cassette pML(C₂AT)²⁹ and HeLa factors TFIIB, TFIIE/F, and RNA polymerase II were complemented with buffer (lane 1), DE52 fraction of native TFIID (lanes 2, 3), amino-octyl fraction of native TFIID (lanes 4, 5) and purified bacterially expressed yeast (lanes 6, 7) or human (lanes 8, 9) TFIID. A DE52 fraction of human activator USF³ was present in lanes 3, 5, 7 and 9. Transcription from the G-free cassette was in the absence of GTP; the upper arrow indicates the full length cassette transcript resulting from specific initiation at +1 whereas the shorter ~ 30 -bp spaced bands (lanes 2-5) are indicative of multiple initiations on the same template²⁹. The lower arrow indicates the position of a labelled DNA fragment added as an internal standard.

METHODS. A human TFIID cDNA (containing nucleotides 1-1,258) in Bluescript SK was transcribed with T7 RNA polymerase and derived RNA was translated in rabbit reticulocyte lysates (Promega) after heat denaturation. Mobility shift assays¹⁵ and primer extension¹⁵ and G-free cassette²⁹ transcription assays were performed as previously described. *S. cerevisiae* and human TFIID cDNAs containing the coding regions were expressed in bacteria, and lysates were prepared as previously described²¹. The recombinant yeast (p27) and human (p38) TATA factors were purified to near homogeneity.

unpublished results). Second, there is a sigma homology region¹⁵, which overlaps the C-terminal direct repeat and introduces an element of asymmetry into the direct repeat structure. This region of TFIID has been implicated in TATA box binding by deletion²⁰ and point mutagenesis studies (T.Y., unpublished results) and we have suggested that it could provide the primary determinants for the specificity of DNA binding. Third, there is a repeat of basic residues¹⁵ (notably lysines) in the central basic core connecting the direct repeats that has the potential for formation of an α -helix with basic residues on one face. Mutagenesis studies indicate that individual basic residues are required neither for DNA binding nor for basal transcription (T.Y., unpublished results), consistent with our earlier suggestion¹⁵ that they might interact with certain types of activators. Fourth, there are myc homologies, noted first by Gasch *et al.*²² for the *Arabidopsis* and yeast proteins, between regions of TFIID and the helix-loop-helix domains of a *myc*-related family of regulatory proteins. As discussed by these authors, this raises the possibility that these regions are important either for homotypic interactions within TFIID or for heterotypic interactions with distinct regulatory factors.

In striking contrast to the conserved C-terminal regions, the N-terminal regions of TFIID differ markedly both in size and sequence (Fig. 4). The human TFIID N terminus lacks the high density of charged residues previously noted²¹ in yeast TFIID, but contains obvious structural features that could be func-

tionally related to similar structural motifs in other regulatory factors. These include (see Fig. 3a): a region containing 34 consecutive glutamines (the Q-run) flanked by two regions (STP-1 and STP-2) rich in serine (S), threonine (T) and proline (P) residues. The latter show a general similarity to Ser, Thr, (Pro)-rich regions in regulatory proteins like SP1²³ and GAL11²⁴, whereas the Ser-Pro, Thr-Pro and Tyr-Pro/Pro-Tyr repeats (usually preceded, followed or interspersed with an Ala, Ile, Leu or Met residue) are reminiscent of the reversibly phosphorylated heptad repeat (Tyr-Ser-Pro-Thr-Ser-Pro-Ser) in the largest subunit of RNA polymerase II²⁵. Similarly the Q-run and adjacent Gln-rich regions resemble polyglutamine and Gln-rich regions present in other factors^{23,24,26} and implicated, in some cases, as activation domains^{23,27}. These regions might well serve as interfaces for intermolecular protein-protein interactions.

Yeast TFIID structure-function studies²⁰ and comparative sequence analyses (Fig. 3) suggest that the human TFIID N terminus is not essential for basal transcription functions. Unlike natural human TFIID^{3,7,10-12}, however, neither bacterially expressed yeast nor human TFIID seem capable of mediating the normal functions of certain activators (like USF) in reconstituted cell-free systems (Fig. 2d; M. Meisterernst, unpublished observations). This suggests that the human N terminus might be involved in mediating regulatory factor interactions, either with TFIID itself or with other general factors, and that these functions might be encoded in separate factors (such as GAL11)

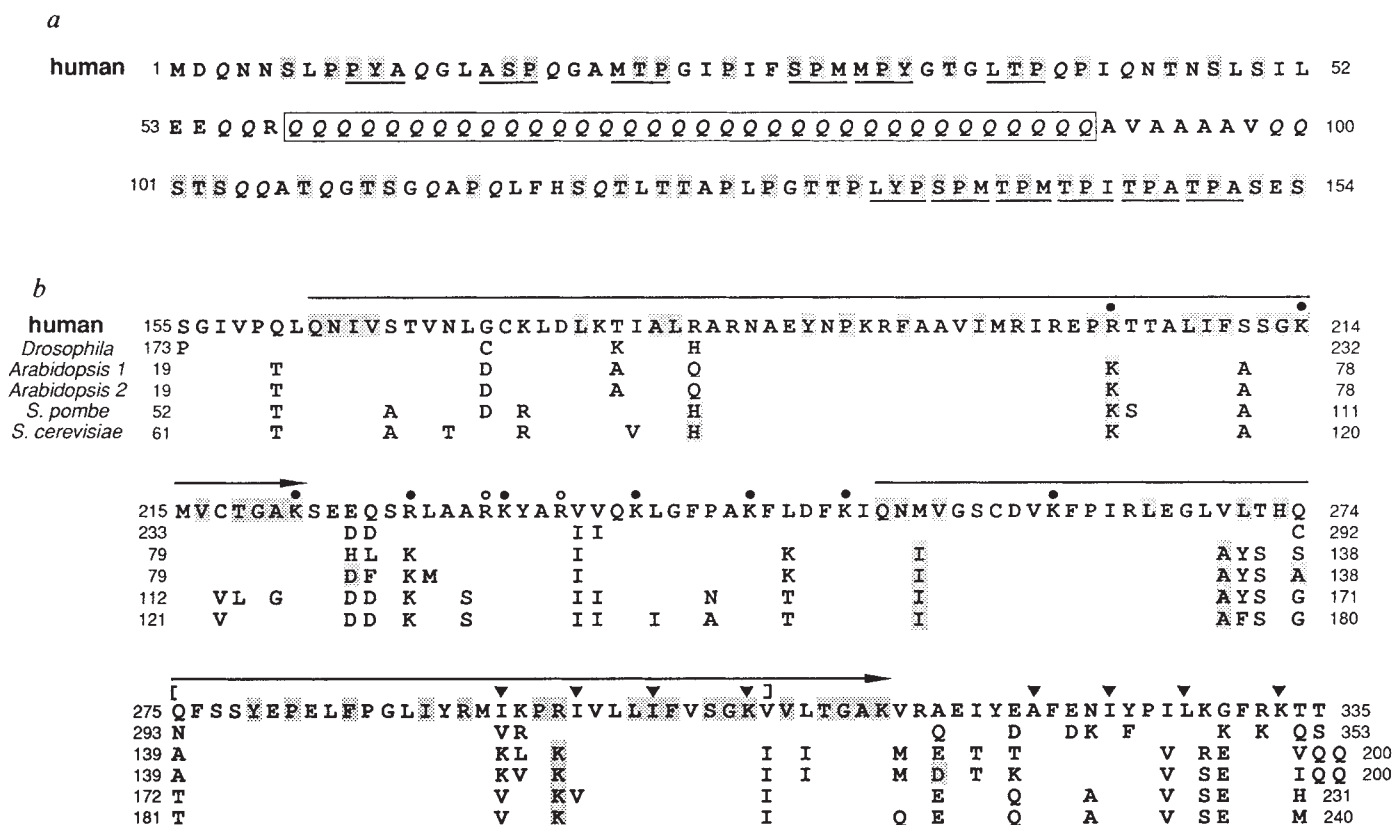


FIG. 3 The human TATA factor consists of a highly conserved TFIID core domain and a unique N-terminal structure. **a**, The amino-acid sequence of the nonconserved N-terminal region of the human TATA factor (single-letter amino-acid code). The potentially interesting primary structure motifs highlighted include a glutamine repeat (boxed) which is flanked by two regions (STP-1 and 2) that are rich in serine, threonine, and proline and characterized by triplets (underlined) containing Ser-Pro, Thr-Pro, and Pro-Tyr/Tyr-Pro pairs preceded or followed by a Met, Ile, Leu, or Ala residue. Alternative arrangements of repeating triplets (such as Pro-Met-Thr, Pro-Ile-Thr) are also possible. **b**, The sequence of the human TFIID core domain is displayed in the top line, whereas the lower lines show only amino acids that differ in the TFIID sequences from other species. Putative structural domains that

are discussed in the text are indicated as follows: (1) direct repeats, long arrows indicate regions containing the two interrupted repeats whose constituent residues are shaded; (2) basic repeat, solid circles (lysines) and open circles (arginines) indicate residues in the central basic core which lie on one face of potential α -helical structures; (3) sigma homology, the region with an overall sequence similarity to the sigma 2.3 and 2.4 domains is indicated by brackets while solid inverted triangles denote residues, in two small regions, that correspond to those most highly conserved in the 2.4 regions of various sigma factors. The regions with sequence similarities to the helix-loop-helix proteins (Gasch *et al.*²²) extend from residues 201 to 220 in direct repeat 1 and from 269 to 314 in direct repeat 2.

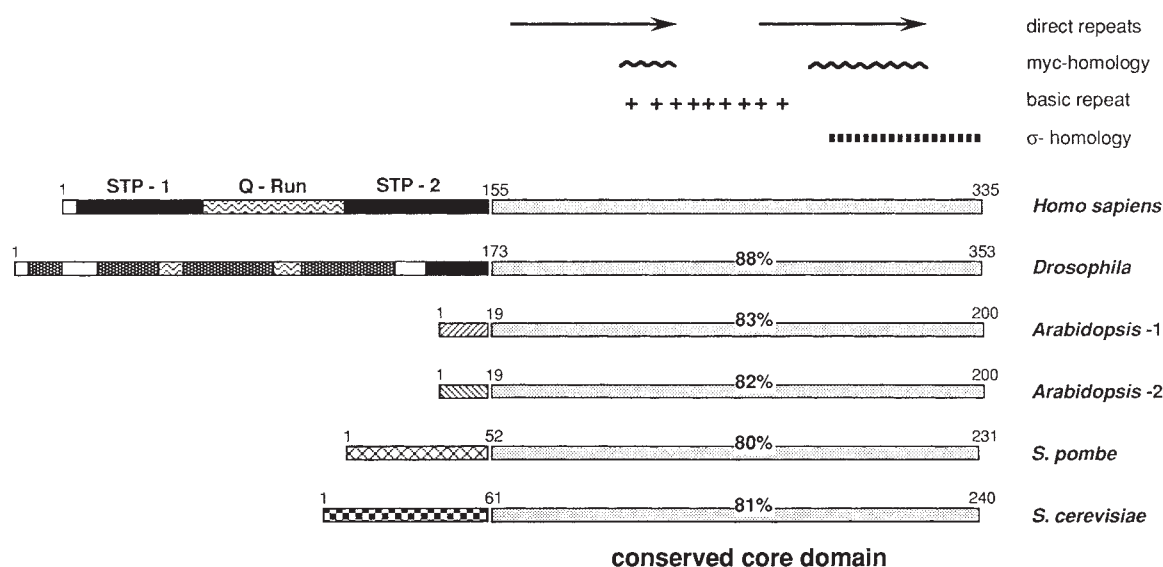


FIG. 4 Evolutionary conservation of the TATA factor primary structure. The degree of sequence identity (relative to human) within the conserved C-terminal core domains of the TFIIDs from human, *Drosophila* (M. Muhich, C. Iida, C. Parker, unpublished results), *Arabidopsis*²², *Schiz. pombe*²¹ and *S. cerevisiae*¹⁵ are summarized. Also indicated are the positions in the core of the structural domains discussed in the text. The N-terminal regions of these proteins differ markedly both in length and amino-acid composition

and sequence, except that the *Drosophila* N terminus shows some sequence similarity (including Gln-rich and Ser, Thr, Pro-rich regions) with the human N terminus. For the *Drosophila* N terminus the black and dark grey boxes represent, respectively, regions with strong and weaker sequence similarities to the human STP regions, and short runs of glutamine (Q-runs) are indicated as for the human N terminus.

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Arabidopsis thaliana contains two genes for TFIID

Alexander Gasch*, Alexander Hoffmann†, Masami Horikoshi†, Robert G. Roeder† & Nam-Hai Chua*‡

* Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, New York 10021-6399, USA

† Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, New York 10021-6399, USA

THE general transcription initiation factor TFIID plays a primary part in the activation of eukaryotic genes transcribed by RNA polymerase II. Binding of TFIID to the TATA box initiates the assembly of other general transcription factors as well as RNA polymerase II at the promoter resulting in a preinitiation complex capable of accurate transcription initiation *in vitro*^{1–3}. Human TFIID has been shown to interact with various regulatory factors^{4–8}. The observation that stimulation of transcription by different *trans*-acting factors is mediated through distinct TATA elements led to the suggestion that different types of TFIID may exist in yeast^{9–11}, humans^{12–15} and plants¹⁶. Here we report the cloning and characterization of two distinct TFIID complementary DNA clones from *Arabidopsis thaliana*. Furthermore, we have found that TFIID from *Arabidopsis* and other organisms shows homology to helix-loop-helix proteins.

Several genomic clones from *Arabidopsis thaliana*, all derived from the same locus, were isolated using the recently isolated yeast TFIID gene^{17–21} as a probe. To obtain related cDNAs we

‡ To whom correspondence should be addressed.

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FIG. 1 Nucleotide sequence of two cDNA classes that encode TFIIID in *Arabidopsis thaliana*. *a*, DNA and deduced amino-acid sequence of At-1 cDNA clones. Solid arrowheads indicate the positions of introns in the sequence of the At-1 genomic clones. Translation start and stop codons are underlined. *b*, DNA and deduced amino-acid sequence of At-2 cDNA clones. Translation start and stop codons are underlined.

METHODS. A 680-base pair (bp) *Nde*I-*Hind*III fragment of the clone pGem11D8^{17,23} containing the majority of the amino-acid coding region of the yeast TFIIID gene was labelled by nick translation and used to screen a genomic library of *A. thaliana*. Hybridization was performed at 20% formamide, 5×SSC, 50 mM Tris-HCl (pH 7.5), 1% SDS, 10× Denhardt's solution and 50 µg ml⁻¹ denatured herring sperm DNA for 16 h at 37 °C. After hybridization, filters were washed in 2×SSC, 1% SDS, twice for 5 min at room temperature and twice for 30 min at 37 °C. This screen yielded four positive clones which were all derived from the same locus. Poly(A)⁺ RNA of whole *A. thaliana* grown under continuous white light was used to construct an oligo(dT)-primed cDNA library in λZap (Stratagene) using the cDNA Synthesis System Plus (Amersham). 750,000 plaques of the primary library were screened under the same conditions as described above for the genomic clones, except that a fragment containing the At-1 TFIIID gene was used as a probe. In total, 20 positive clones were obtained in this screen. After *in vivo* excision, nine of these clones, as well as subclones of the genomic fragments hybridizing with the yeast TFIIID probe, were subjected to double-stranded sequencing using the Sequenase sequencing kit (USB). Sequence data were analysed with the DNASIS and PROSIS programs (Hitachi) on an IBM PS/2 computer.

<i>a</i>		CG CAG ATC ACA AAT																				14
1	15	CTC	ATC	CCC	AGA	GAG	AGA	ACC	CAG	AGA	GCG	ATA	TTG	AAA	ATC	AAA	ACT	CTC	TCC	TTT	ATA	74
	75	TAT	AAT	CCC	AAT	TTA	CAA	ATC	TCT	TTC	CCT	CTC	TAA	AAA	TTT	CTT	ATC	TTT	GTA	TAA	AAA	134
	135	GCC	TTC	TCC	TTT	TTC	AAA	TCA	TTC	ACC	TTC	CTT	TCG	CCT	TTT	CGG	GGG	AAT	TTC	TTC	CAA	194
	195	TCT	GTT	GGG	TTT	ACG	AAA	CCC	TAG	ATT	TCG	AAA	TTC	CGG	ATT	GCT	AAC	TAG	CGA	AAG	AGA	254
	255	<u>ATG</u>	<u>GCT</u>	<u>GAT</u>	<u>CAA</u>	<u>GGA</u>	<u>ACG</u>	<u>GAA</u>	<u>GGG</u>	<u>AGC</u>	<u>CAG</u>	<u>CCA</u>	<u>GTT</u>	<u>GAC</u>	<u>CTT</u>	<u>ACT</u>	<u>AAA</u>	<u>CAT</u>	<u>CCT</u>	<u>TCA</u>	<u>GGG</u>	314
1		M	A	D	Q	G	T	E	G	S	Q	P	V	D	L	T	K	H	P	S	G	20
	315	ATT	GTT	CCT	ACT	CTT	CAA	AAT	ATT	GTC	TCA	ACA	GTG	AAC	TTA	GAC	TGC	AAG	CTT	GAT	CTT	374
21		I	V	P	T	L	Q	N	I	V	S	T	V	N	L	D	C	K	L	D	L	40
	375	AAA	GCC	ATA	GCT	TTG	CAA	GCT	AGG	AAT	GCT	GAA	TAT	AAC	CCC	AAG	CGT	TTT	GCT	GCT	GTA	434
41		K	A	I	A	L	Q	A	R	N	A	E	Y	N	P	K	R	F	A	A	V	60
	435	ATC	ATG	AGG	ATC	AGA	GAG	CCA	AAG	ACC	ACA	GCG	TTA	ATT	TTT	GCT	TCT	GGG	AAA	ATG	GTG	494
61		I	M	R	I	R	E	P	K	T	T	A	L	I	F	A	S	G	K	M	V	80
	495	TGT	ACC	GGA	GCT	AAA	AGT	GAA	CAT	CTT	TCA	AAG	CTT	GCT	GCA	AGA	AAG	TAT	GCT	CGG	ATT	554
81		C	T	G	A	K	S	E	H	L	S	K	L	A	A	R	K	Y	A	R	I	100
	555	GTT	CAA	AAG	CTT	GGC	TTT	CCT	GCA	AAG	TTC	AAG	GAT	TTT	AAG	ATA	CAG	AAC	ATT	GTA	GGC	614
101		V	Q	K	L	G	F	P	A	K	F	K	D	F	K	I	Q	N	I	V	G	120
	615	TCA	TGT	GAT	GTC	AAA	TTC	CCC	ATT	AGG	CTT	GAA	GGT	CTT	GCA	TAC	TCT	CAT	AGT	GCT	TTC	674
121		S	C	D	V	K	F	P	I	R	L	E	G	L	A	Y	S	H	S	A	F	140
	675	TCA	AGT	TAC	GAG	CCT	GAG	CTA	TTT	CCA	GGA	TTG	ATA	TAT	AGG	ATG	AAA	CTT	CCA	AAG	ATT	734
141		S	S	Y	E	P	E	L	F	P	G	L	I	Y	R	M	K	L	P	K	I	160
	735	GTA	CTG	CTT	ATT	TTT	GTG	TCA	GGA	AAG	ATT	GTT	ATA	ACT	GGA	GCC	AAG	ATG	AGA	GAA	GAG	794
161		V	L	L	I	F	V	S	G	K	I	V	I	T	G	A	K	M	R	E	E	180
	795	ACT	TAT	ACC	GCA	TTT	GAG	AAT	ATC	TAC	CCA	GTT	CTT	AGA	GAA	TTC	AGG	AAG	GTC	CAG	CAA	854
181		T	Y	T	A	F	E	N	I	Y	P	V	L	R	E	F	R	K	V	Q	Q	200
	855	<u>TAA</u>	<u>TAG</u>	<u>TAT</u>	<u>AGC</u>	<u>CTA</u>	<u>CCA</u>	<u>TAT</u>	<u>TGA</u>	<u>AGG</u>	<u>CAA</u>	<u>AAA</u>	<u>CCA</u>	<u>TGT</u>	<u>TCA</u>	<u>CGT</u>	<u>TTG</u>	<u>GAT</u>	<u>TGC</u>	<u>CAT</u>	<u>GAA</u>	914
915		CTA	GAG	ATC	TCG	GGT	GTT	GGC	AGG	CGC	TTG	GTG	GGG	TTA	TAT	TCC	TTG	GAC	AAG	AAC	TTG	974
	975	AAA	TGA	TTC	AGA	TTC	AAA	ACC	AGA	AAT	TGT	AAA	TAG	AGA	ATG	GAG	ATG	CCA	ATA	AAT	CTC	1034
1035		TTT	TCT	TTT	TGT	TCT	AAA	AAT	GTC	TTC	CAA	GTA	ATC	TTG	TTT	CAT	GTT	CTG	TTT	GTT	TCA	1094
	1095	GTG	AAC	AAT	ACC	CAA	TCA	TTA	AAG	AGT	TAC	TTA	GTT	CTT	TGC	TAA	CTG	TTC	TGT	AAT	AAG	1154
1155		ACA	CCA	AGT	CTG	TTT	GCC	CTT	TAA	TAT	ACT	CTT	TCA	AGC								1193
<i>b</i>		CA CAG ATC TAA AAA CCA GAA GCG AGA GAA ACA AAT TCA CTC TCC CCT																				47
	48	TAT	ATA	AGC	ACC	GAT	TTA	TAA	ATC	TTT	TTC	CCT	CTT	CGA	TTC	TCA	ATT	CTT	TGT	ATA	AAA	107
	108	GGC	TTC	TCC	TTT	TCT	CAA	TTC	TTC	GGC	TTC	CTT	TCG	CCC	AAA	CTC	TTC	CCT	CGA	ATC	TTT	167
	168	CCT	TCT	CGT	CTT	AAA	GCT	ACG	AAA	CCC	TAG	ATT	TCG	GAT	TTC	TTC	GCT	ATC	CAA	AGA	AGA	227
	228	<u>ATG</u>	<u>ACT</u>	<u>GAT</u>	<u>CAA</u>	<u>GGA</u>	<u>TTG</u>	<u>GAA</u>	<u>GGG</u>	<u>AGT</u>	<u>AAT</u>	<u>CCA</u>	<u>GTT</u>	<u>GAT</u>	<u>CTT</u>	<u>AGC</u>	<u>AAG</u>	<u>CAT</u>	<u>CCT</u>	<u>TCA</u>	<u>GGG</u>	287
1		M	T	D	Q	G	L	E	G	S	N	P	V	D	L	S	K	H	P	S	G	20
	288	ATT	GTT	CCT	ACT	CTT	CAA	AAC	ATT	GTC	TCC	ACG	GTG	AAC	TTA	GAC	TGC	AAG	CTA	GAT	CTT	347
21		I	V	P	T	L	Q	N	I	V	S	T	V	N	L	D	C	K	L	D	L	40
	348	AAA	GCC	ATA	GCT	TTG	CAG	GCT	CGG	AAT	GCT	GAA	TAT	AAT	CCC	AAG	CGT	TTT	GCT	GCG	GTG	407
41		K	A	I	A	L	Q	A	R	N	A	E	Y	N	P	K	R	F	A	A	V	60
	408	ATA	ATG	AGG	ATC	AGA	GAA	CCG	AAG	ACT	ACA	GCA	TTA	ATA	TTC	GCC	TCA	GGG	AAA	ATG	GTC	467
61		I	M	R	I	R	E	P	K	T	T	A	L	I	F	A	S	G	K	M	V	80
	468	TGT	ACT	GGA	GCT	AAG	AGC	GAG	GAC	TTT	TCG	AAG	ATG	GCT	GCT	AGA	AAG	TAT	GCT	AGG	ATT	527
81		C	T	G	A	K	S	E	D	F	S	K	M	A	A	R	K	Y	A	R	I	100
	528	GTG	CAG	AAA	TTG	GGA	TTC	CCT	GCA	AAA	TTC	AAG	GAT	TTC	AAG	ATT	CAG	AAT	ATT	GTA	GGT	587
101		V	Q	K	L	G	F	P	A	K	F	K	D	F	K	I	Q	N	I	V	G	120
	588	TCT	TGT	GAT	GTC	AAA	TTC	CCT	ATA	AGA	CTT	GAA	GGT	CTT	GCT	TAC	TCT	CAC	GCT	GCT	TTC	647
121		S	C	D	V	K	F	P	I	R	L	E	G	L	A	Y	S	H	A	A	F	140
	648	TCA	AGT	TAT	GAG	CCC	GAG	CTC	TTC	CCA	GGG	CTG	ATT	TAT	AGG	ATG	AAA	GTC	CCA	AAA	ATC	707
141		S	S	Y	E	P	E	L	F	P	G	L	I	Y	R	M	K	V	P	K	I	160
	708	GTC	CTT	CTA	ATC	TTT	GTC	TCT	GGG	AAG	ATC	GTA	ATA	ACA	GGA	GCC	AAG	ATG	AGA	GAT	GAG	767
161		V	L	L	I	F	V	S	G	K	I	V	I	T	G	A	K	M	R	D	E	180
	768	ACC	TAC	AAA	GCC	TTT	GAG	AAT	ATA	TAC	CCC	GTG	CTC	TCG	GAA	TTC	AGA	AAG	ATA	CAG	CAA	827
181		T	Y	K	A	F	E	N	I	Y	P	V	L	S	E	F	R	K	I	Q	Q	200
	828	<u>TAG</u>	<u>GTC</u>	<u>ACG</u>	<u>GAT</u>	<u>TTG</u>	<u>TTC</u>	<u>CCT</u>	<u>GCA</u>	<u>AAA</u>	<u>CTA</u>	<u>GTT</u>	<u>GTG</u>	<u>CGG</u>	<u>TCT</u>	<u>TAG</u>	<u>CCC</u>	<u>CTT</u>	<u>GGA</u>	<u>GTT</u>	<u>GCT</u>	887
888		AAA	GCT	TGC	TGA	GAA	TTT	TGC	CCT	TGA	ACA	AAG	GCT	TTC	ACA	GTA	GCT	AGA	CTC	TCA	CCT	947
	948	TGT	GTT	TTG	GTT	CAT	AAT	ATA	ACA	TTG	TAT	ATA	CAC	AAT	GGA	GAT	TCT	AAA	GAC	AAT	TCT	1007
1008		TCG	GTT	CTA	TTT	TTT	TTC	TTT	TTC	TTT	CCA	AGA	TAT	GTC	TTA	CAT	GTA	TGT	TGC	TGT	AGT	1067
1068		GTC	CTC	ACA	TTT	CCA	CTT	ATG	TTA	CAA	GTA	GAA	CCT	TAA	CTC							1109

screened a primary *A. thaliana* cDNA library under low stringency hybridization conditions using one of the genomic clones as a probe. We obtained 20 positive clones (out of 7.5×10^4) of which nine were analysed further by sequencing. Seven of these cDNA clones (*A. thaliana* type 1, At-1) correspond to the isolated *A. thaliana* genomic clones (Fig. 1a), whereas the remaining two cDNA clones (*A. thaliana* type 2, At-2) contain a very similar but clearly distinct sequence (Fig. 1b). Genomic Southern analysis using the At-1 and At-2 cDNA clones as probes confirms that there are at least two closely related genes present in the *Arabidopsis* genome (data not shown). Northern analysis using gene-specific probes reveals that the At-1 and At-2 messenger RNAs are 1.4 kilobases (kb) and 1.3 kb in size, respectively and that they are present in roughly equal amounts in whole light-grown *Arabidopsis* plants (data not shown). A sequence comparison between the At-1 cDNA and genomic clones reveals the presence of nine introns, the positions of which are indicated in Fig. 1a. The longest open reading frame in both types of cDNA clones encodes a protein of 200 amino acids, highly homologous to, but 40 amino acids smaller than, yeast TFIID. The calculated relative molecular mass (M_r) is 22,395 for *Arabidopsis* TFIID-1 and 22,367 for *Arabidopsis* TFIID-2.

To determine whether both cDNA clones encode a functional TFIID we tested TATA box-binding and basal transcription activities of *in vitro* expressed proteins. For At-1 as well as At-2 a protein of M_r 24,000 in size was obtained (Fig. 2a), matching the calculated size. Both proteins bind specifically to the adenovirus major late promoter in gel retardation assays (Fig. 2b). Both types of TFIID give three shifted complexes (termed 1 and 1', 2, 3), suggesting that proteins in the reticulocyte lysate either modify or interact with the two proteins. Interestingly, complex 1 formed in the presence of the TFIID-1 is slower in mobility than complex 1' formed in the presence of the TFIID-2, although both proteins have approximately the same molecular weight. This could indicate an interaction of TFIID-1 with proteins present in the reticulocyte lysate. Alternatively, the complexes of TFIID-1 and TFIID-2 bound to DNA could have different structures as a result of either a difference in the secondary structure of the two proteins or a difference in the way they bind to DNA.

To study further the function of both types of *Arabidopsis* TFIID, we expressed the two TFIID cDNAs in *Escherichia coli* (Fig. 2c). Crude extracts of cells expressing these proteins were then tested for TFIID activity in a reconstituted human *in vitro* transcription system containing RNA polymerase II and the basic transcription factors except TFIID. We used a template that contains the adenovirus major late promoter fused to a guanosine-free cassette²². Extracts of cells expressing TFIID-1 or TFIID-2 are both able to confer basal transcription in this assay whereas extracts of cells containing the expression vector alone have no activity (Fig. 2d). Taken together, these results clearly demonstrate that both cDNA clones encode functional TFIID as both proteins exhibit a specific binding activity to the TATA box and can substitute for the human TFIID to activate basal transcription *in vitro*.

Figure 3 compares the deduced amino-acid sequences of *Arabidopsis* TFIID-1 and TFIID-2. Out of 200 residues, 187 (93.5%) are identical between the two proteins. The N-terminal region (amino acids 1–18) is less conserved (75% identity) than the rest (19–200) of the protein (95% identity). Figure 3 also compares the sequences of both *Arabidopsis* TFIID proteins with that of the yeast TFIID. The N-terminal domain is completely divergent in sequence and smaller in size (18 compared with 60 residues) between *Arabidopsis* and yeast TFIID. Moreover, the N-terminal domain of yeast TFIID is dispensable for basal level transcription²³ suggesting that it is involved in contacting species-specific regulatory factors. The large C-terminal domains, however, are highly conserved between the *Arabidopsis* and yeast TFIID proteins (85% amino acid sequence identity). The high sequence conservation suggests that this domain is involved in the highly conserved TFIID properties, such as TATA box-binding and interaction with the general transcription machinery. Indeed, these properties have been mapped to the C-terminal residues 63–240 of yeast TFIID²³. This TFIID domain harbours several previously described sequence motifs, all highly conserved between the *Arabidopsis* and yeast TFIID proteins: a domain rich in basic residues¹⁷, a region of similarity to bacterial sigma factors¹⁷ and a recently discovered direct repeat²¹. Possible functions of these sequence motifs, which are schematically shown in Fig. 4, are discussed in the accompanying manuscript²⁴. Recently it was shown that

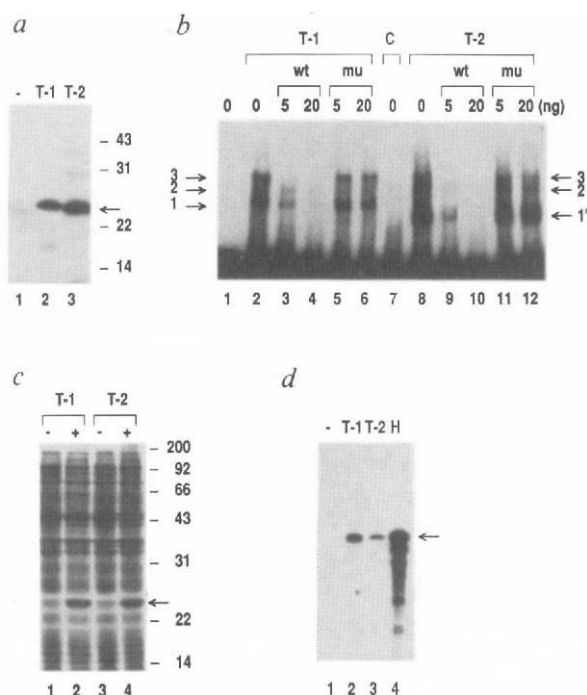


FIG. 2 Functional analysis of *Arabidopsis* TFIID-1 and TFIID-2. *a*, SDS-PAGE of [³⁵S]methionine-labelled TFIID-1 (T-1) and TFIID-2 (T-2) obtained by *in vitro* translation. Samples contained reticulocyte lysate programmed with no (–) RNA (lane 1), with At-1 RNA (lane 2), with At-2 RNA (lane 3). The arrow indicates the TFIID proteins. *b*, Site-specific DNA binding of TFIID-1 (T-1) and TFIID-2 (T-2). An adenovirus major late promoter fragment¹⁷ was used for the gel shift assay¹⁷. The reactions included: no protein (lane 1), reticulocyte lysate programmed with At-1 RNA (lanes 2–6), with no (C) RNA (lane 7), with At-2 RNA (lanes 8–12). Binding was competed with 5 ng (lanes 3, 9), and 20 ng (lanes 4, 10) of wild-type (wt) TATA box sequence (TATAAAA) competitor DNA¹⁷ or with 5 ng (lanes 5, 11), and 20 ng (lanes 6, 12) of mutant (mu) TATA box sequence (TAGAGAA) competitor DNA¹⁷. *c*, SDS-PAGE of TFIID-1 (T-1) and TFIID-2 (T-2) expressed in *E. coli*. The lanes contain *E. coli* extracts carrying pET3a²⁶ with At-1 cDNA (lanes 1, 2), or At-2 cDNA (lanes 3, 4), either before (–) or after (+) induction with IPTG. The arrow indicates the position of TFIID. *d*, Activation of *in vitro* transcription by TFIID-1 (T-1) and TFIID-2 (T-2) in a reconstituted HeLa *in vitro* transcription system. Added were extracts of induced cells containing pET3a²⁶ without (–) insert (lane 1), with At-1 cDNA (lane 2) or At-2 cDNA (lane 3). In lane 4 partially purified human (H) TFIID¹ was added. The arrow indicates transcripts of 380 nucleotides.

METHODS. The At-1 and At-2 cDNA clones were transcribed *in vitro* using T3 RNA polymerase and T7 RNA polymerase (Stratagene), respectively. *In vitro* translation, SDS-PAGE analysis and gel retardation assays were performed as described¹⁷. Expression in *E. coli*, preparation of crude extracts and transcription assays were performed as described²⁷.

can be distinguished by their affinity to one or more of these components. The positions of amino-acid exchanges between TFIID-1 and TFIID-2 could provide some clue to where possible functional differences might be encoded. Further experiments are needed to determine exactly to what degree both factors differ in function and to pinpoint the residues that are important in these differences. □

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Spontaneous shuffling of domains between introns of phage T4

Mary Bryk*† & Marlene Belfort*‡§

* Wadsworth Center for Laboratories & Research, New York State Department of Health, Empire State Plaza, PO Box 509, Albany, New York 12201-0509, USA

† Department of Microbiology & Immunology, Albany Medical College, 47 New Scotland Avenue, Albany, New York 12208, USA

‡ School of Public Health, State University of New York at Albany, Empire State Plaza, Albany, New York 12201, USA

THE three self-splicing introns in phage T4 (in the *td*, *sunY* and *nrdB* genes) (Fig. 1a) each have the conserved group I catalytic RNA core structure (Fig. 1b), out of which is looped an open reading frame¹. Although the core sequences are very similar (~60% identity), the open reading frames seem to be unrelated. Single crossover recombination events between homologous core sequences in the closely linked *td* and *nrdB* introns have led to 'exon shuffling'². Here we describe spontaneous double crossovers between the unlinked *td* and *sunY* introns that result in shuffling of an intron structure element, P7.1 (refs 3 and 4). The intron domain-switch variants were isolated as genetic suppressors of a splicing-defective P7.1 deletion in the *td* intron. This unprecedented example of suppression through inter-intron sequence substitution indicates that the introns are in a state of genetic flux and implies

the functional interchangeability of the two analogous but non-identical P7.1 elements. The implications of such recombination events are discussed in the light of the evolution of the introns themselves as well as that of their host genomes.

To address questions of intron function and evolution we have isolated Td⁺ pseudorevertants from Td⁻ phage containing stable splicing-defective intron mutations. The Δ P7.1 deletion (Fig. 1c), which was introduced into a functional 265-nucleotide (nt) *td* mini-intron⁵ (Fig. 1b) and shown to inhibit splicing (R. Schroeder, unpublished observations), was crossed into the phage, allowing selection for Td⁻ plaques^{6,7}. Subsequently, Td⁺ plaques^{7,8}, which arose spontaneously from these T4td Δ P7.1 phage at a frequency of $\sim 10^{-7}$, were isolated and characterized by plaque hybridization (not shown) and sequence analysis (Fig. 2). Remarkably, four independent Td⁺ pseudorevertants had acquired the P7.1 element from the *sunY* intron (Figs 1c and 2). Although the P7.1 elements of *td* and *sunY* differ (stems of 4 versus 5 base pairs (bp) and loops of 4 versus 10 nt, respec-

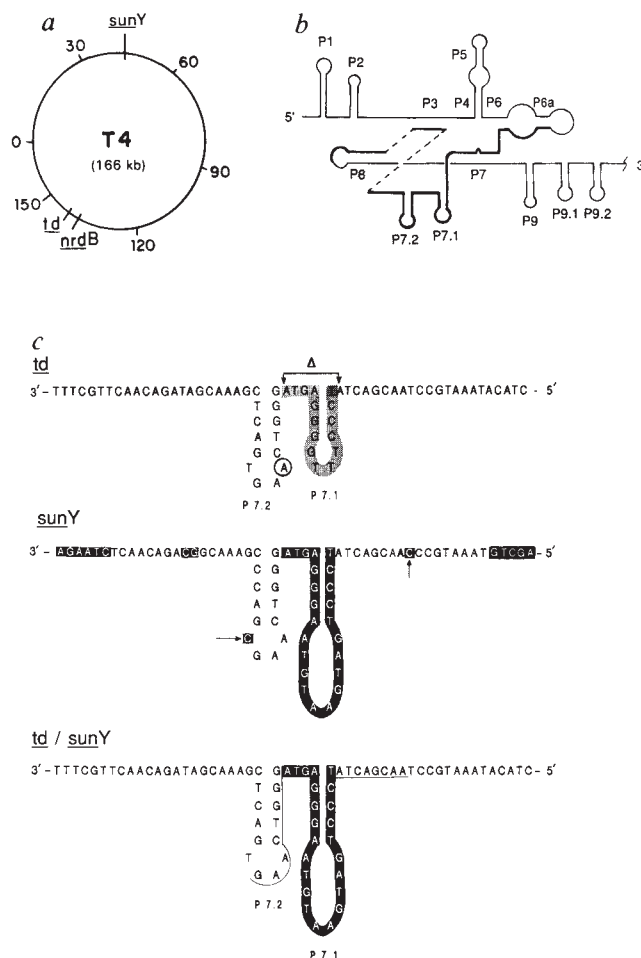


FIG. 1 a, Map of the circularly permuted T4 genome. The positions of the intron-containing *td*, *nrdB* and *sunY* genes are shown on the 166-kilobase (kb) map, calibrated in kb. b, Secondary structure map of the *td* intron. The pairing elements (P1-P9.2) are shown^{1,4}, with the endonuclease-encoding ORF—naturally looped out of P6a—deleted⁵, as it is in all *td*-containing phage and plasmids used in this work. The bold line indicates the sequences presented in c. c, Sequences of the P3-P7 region. The *td* sequence (top) shows the P7.1 deletion in T4td Δ 7.1 shaded and bracketed by arrows (Δ). In the *sunY* sequence (middle), nucleotides present in *sunY* but not in T4td Δ 7.1 are shown on a black background. Arrows point to the nucleotides that define the recombination boundary. The T4td-*sunY*7.1 hybrid (bottom) has the nucleotides over which the double crossover event occurred marked by thin lines. These homologies are shown here as 8 nt on each side of P7.1, but could be drawn as 6 nt (5') and 10 nt (3') as there is a redundant AT sequence on both sides of P7.1. The circled A in the *td* sequence represents the 3' residue in the autoradiographs shown in Fig. 2.

§ To whom correspondence should be addressed.

RNA/messenger RNA ratios of *td* transcripts from phage-infected cells, using a primer-extension assay. Thus, T4*td*Δ7.1 was shown to be splicing-defective, whereas T4*td-sunY*7.1 was shown to undergo high levels of accurate splicing (Fig. 4a). To assess self-splicing proficiency, the hybrid intron was crossed into an *in vitro* transcription vector. Plasmids containing the wild-type *td* or *td-sunY*7.1 introns conferred a Thy⁺ (Td⁺) phenotype on Thy⁻ cells¹⁰, whereas those with the *td*Δ7.1 intron did not. Accordingly, *td*Δ7.1 precursors did not function *in vitro*, whereas *td*⁺ and *td-sunY*7.1 pre-mRNA spliced efficiently (Fig. 4b). Although subtle differences in splicing activity have not been ruled out, these results suggest that the different P7.1 elements of the *td* and *sunY* introns are functionally interchangeable.

The type of ectopic crossover described here would influence only the intron core structure and therefore splicing. However, parallel events involving sequences flanking an open reading frame (ORF) could have a profound influence on the composition and ultimate fate of the intron. As both the *td* and *sunY* ORFs encode endonucleases that promote intron mobility^{7,12,13}, recombinational 'ORF-shuffling' could result in either limiting or extending intron spread.

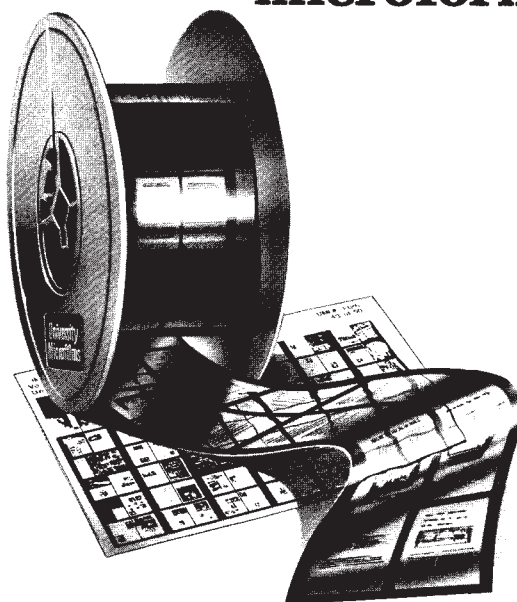
Double crossovers between separated elements extend the range of viable recombinational possibilities because they leave undisturbed the regions between the homologous elements. By contrast, single crossovers lead to major genetic rearrangements between repeated elements¹⁴ and are therefore confined to introns that span non-essential regions. Such events are exemplified by hybrids of the closely linked *td* and *nrdB* introns, which have undergone deletion of the dispensable sequences between them². Because double crossovers introduce discrete genetic replacements in the homologous elements they can occur between distant loci without threatening viability, as demonstrated here for the unlinked *td* and *sunY* introns. Because as few as five homologous nucleotides are required at an inter-intron crossover locus (M.B. and M.B., unpublished observations), similar double recombination events may also facilitate exchanges involving exons (with one crossover within homologous introns, the other in flanking exons). Although the latter events remain to be demonstrated, the ability of unlinked introns to facilitate recombination over wide genetic distance is clear. The possibility that such crossovers might occur interchromosomally adds further interest to the hypothesis that introns act as 'recombinogens' that increase the opportunities for genetic exchange and thereby speed the evolution of genomes¹⁵. Boosting the evolutionary fitness of the phages may indeed provide the rationale for the maintenance of introns, which are without an apparent phenotype¹⁶, in the reproductively efficient phages. Nevertheless, inter-intron sequence translocation must influence evolution of the introns themselves. □

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